

GENETIC ANALYSIS OF MUTATIONS AFFECTING THE

INITIATION OF YEAST SPORULATION

BY

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For my parents...

A. D. RAMAGE





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## ABSTRACT

The *spd* (sporulation derepressed) mutants of *Saccharomyces cerevisiae* appear to have lost some of the nitrogen source repression of sporulation but remain subject to glucose repression. In media containing acetate, glycerol or pyruvate as the sole carbon source, these mutants sporulate profusely, a phenotype referred to as hypersporulation (Dawes and Calvert, 1984). This hypersporulation phenotype is also characteristic of mutants in the cAMP signal pathway which have low intracellular levels of cAMP e.g. *cdc25*, *ras2*, *cdc35*, yet the *spd1* mutants are not allelic to any of these genes.

A 4.8 kb DNA sequence on a multicopy plasmid was isolated by its ability to restore growth and inhibit hypersporulation of *spd1* mutants on YEPG medium. Using the technique of Tn5 transposon mutagenesis, an area of 1.7 - 2.6 kb within the sequence was shown to be responsible for the above effects. The sequence hybridized to chromosome VIII indicating that it was an extragenic suppressor of the *SPD1* gene, which is situated on chromosome XV. Cells disrupted within the complementing region of the sequence grew more slowly than the corresponding wild-type on YEPD media. On complete media containing ethanol, glycerol or acetate as the sole carbon source, the disruptant did not grow at 30°C but grew at 26°C although at a slower rate than the corresponding wild-type. Diploids homozygous for the above disruption sporulated to a much lesser extent than the corresponding wild-type cells and showed a significantly slower rate of respiration than the wild-type on both YEPD and YEPG media.

DNA sequence analysis of the complementing region of the above sequence showed that it was identical to the *SCH9* gene which was isolated by Toda *et al.*, (1988) as a suppressor of the *cdc25-1* mutation. Toda and colleagues found that the *SCH9* gene was a protein kinase structurally and functionally





related to cAMP-dependent protein kinase catalytic subunits. It is proposed that the SCH9 protein kinase may be involved in a cAMP-independent signal pathway.

A second suppressor of a *spd1* mutant was isolated on a 15 kb insert of a centromeric plasmid gene bank. This sequence has been partially characterized. It was found to restore growth and inhibit hypersporulation of *spd1* mutants on YEPG medium but, unlike *SCH9*, did not suppress the temperature sensitive *cdc25-5* mutation.

The above results are discussed in terms of current knowledge of sporulation control and second messenger pathways in yeast.





## LIST OF ABBREVIATIONS

$A_x$	: absorbance of a sample, through a 1 cm light path at wavelength X nm
ADP	: adenosine 5'-diphosphate
Amp <sup>R/S</sup>	: ampicillin resistance/sensitivity
ATP	: adenosine 5'-triphosphate
bp	: base pairs
BSA	: bovine serum albumin
Ci	: Curie (unit of radioactivity)
cpm	: counts per minute
d	: day(s)
DMSO	: dimethylsulphoxide
DNA	: deoxyribonucleic acid
DNase	: deoxyribonuclease
dNTP	: deoxynucleoside 5'-triphosphate (dATP, dCTP, dGTP, dTTP)
ddNTP	: dideoxynucleoside 5'-triphosphate (ddATP, ddCTP, ddGTP, ddTTP)
DTT	: dithiothreitol
<i>E. coli</i>	: <i>Escherichia coli</i>
EDTA	: ethylenediaminetetraacetic acid
g	: centrifugal field
h	: hour(s)
kb	: kilobase or kilobase pair
kDal	: kilodalton (molecular weight)
K <sub>n</sub> <sup>R/S</sup>	: kanamycin resistance/sensitivity
min	: minute(s)
NADP(NADP <sup>+</sup> , NADPH)	: nicotinamide-adenine dinucleotide phosphate (oxidised, reduced)
<sup>32</sup> P	: phosphorus-32





PEG	: polyethylene glycol
RNA	: ribonucleic acid
RNase	: ribonuclease
<i>S. cerevisiae</i>	: <i>Saccharomyces cerevisiae</i>
SDS	: sodium dodecyl sulphate
sec	: second(s)
<i>spd</i>	: sporulation derepressed
TE buffer	: 10 mM Tris-HCl; 1mM EDTA; pH 8
Tet <sup>R</sup> /S	: tetracycline resistance/sensitivity
Tris	: 2-amino-2-hydroxymethylpropane-1,3-diol
UV	: ultra-violet light
V	: volt
vol	: volume
X-Gal	: 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactoside





# INTRODUCTION







Cell differentiation is of central importance to all eukaryotic organisms, yet specific knowledge of the genetic and biochemical control of developmental events is still very limited. The process of sporulation in the budding yeast *Saccharomyces cerevisiae*, which includes meiosis and ascospore development, provides a relatively simple model system for the investigation of eukaryotic differentiation and for this reason has been the focus of numerous studies. Moreover, as an experimental system, *S. cerevisiae* has all the technical advantages of a well-studied microbial system.

### **LIFE CYCLE OF THE YEAST *Saccharomyces cerevisiae***

The yeast *S. cerevisiae* exists in both haploid and diploid forms and the life cycle of this organism consists of proliferation of cells, mating and meiosis (Fig. 1). Both haploids and diploids can undergo cell division by budding and when diploids are heterozygous ( $a/\alpha$ ) for the mating-type alleles, they will undergo meiosis when starved, to form four spherical haploid products known as ascospores. When favourable conditions occur, the haploid ascospores will begin vegetative growth. If  $a$  and  $\alpha$  cells are mixed, they undergo mating to produce a transient heterokaryon. Nuclear fusion then occurs and a heterozygote is formed which may divide vegetatively through budding.

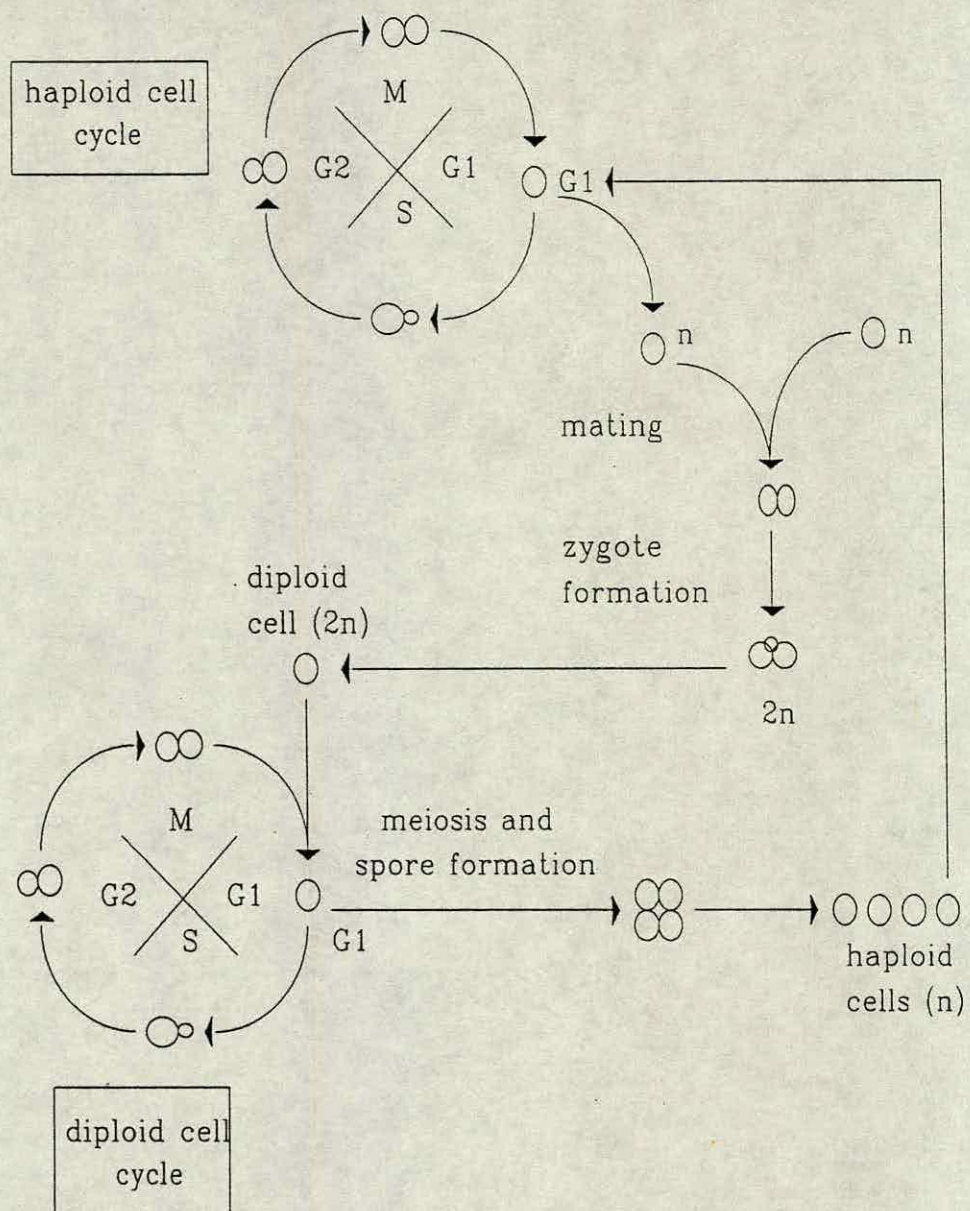
*S. cerevisiae* has homothallic and heterothallic life cycles which will both be described.

### **Proliferation: the Mitotic Cell Cycle**

The process of cell proliferation occurs by budding (Pringle and Hartwell, 1981) and is referred to as the mitotic cell cycle or cell division cycle. Given enough nutrients the yeast population will double in number about every 100 minutes. The mechanism of budding involves a mother cell producing an







**Figure 1:** The life cycle of heterothallic strains of *Saccharomyces cerevisiae*. G1, G2, S and M refer to the phase of the cell division cycle.







initially small protuberance (bud emergence) which gives rise to a daughter cell after chromosome duplication. If the cells run out of nutrients they may arrest as unbudded cells in the G1 phase of the life cycle where they survive well and resume growth when nutrients are available.

### **Heterothallic life cycle - mating and sporulation**

In *S. cerevisiae*, mating-type is determined by two different alleles of the same locus *MAT*, referred to as **a** and  $\alpha$ . When haploid cells of the **a** and  $\alpha$  cell types contact each other they will mate with nearly 100% efficiency. The product of mating is called a zygote and has a distinctive shape. The diploid cells proliferate by budding to give rise to a specialized cell type - a diploid **a**/ $\alpha$ . This cell type cannot mate with **a** or  $\alpha$  cells but can undergo meiosis giving rise to four haploid progeny each encased in a spore coat and all four enclosed in an ascus.

Haploid **a** and  $\alpha$  cells produce specific signalling molecules and receptor systems. Cells of each haploid type produce a pheromone; **a** cells produce **a**-factor, a peptide of 12 amino acids (Betz *et al.*, 1987), while  $\alpha$  cells produce  $\alpha$ -factor which codes for a peptide of 13 amino acids (Stötzler *et al.*, 1976). The mating factors cause cells to arrest in the G1 phase of the cell division cycle; growth is inhibited and mating is stimulated, since the mating factors activate synthesis of proteins necessary for cell and nuclear fusion (McCaffrey *et al.*, 1987; Rose *et al.*, 1986). The precise sequence of events is not yet known.

Gene sets that are active in different cell types are determined by genes of the mating type locus - these genes act as master regulatory elements governing cell specialization. Haploid  $\alpha$  cells produce  $\alpha$  factor and a receptor for **a** factor under the control of an activator protein MAT $\alpha$ 1 which activates transcription of the corresponding  $\alpha$ -specific genes (Sprague *et al.*, 1983). The same cells also contain a repressor protein MAT $\alpha$ 2 which blocks transcription of corresponding **a**-specific genes (Hartig *et al.*, 1986; Wilson and Herskowitz,







1984).  $\alpha 1$  and  $\alpha 2$  are DNA-binding proteins (Bender and Sprague, 1987; Johnson and Herskowitz, 1985; Keleher *et al.*, 1988).

Haploid **a** cells contain no activator and no suppressor proteins (Strathern, 1981) but the **a1** product of *MATa* has an important role in **a**/ $\alpha$  cells (Kassir and Simchen, 1976).

**a**/ $\alpha$  cells have no receptor and no mating pheromone. The  $\alpha 2$  product represses synthesis of products characteristic of **a** cells. In combination with the **a1** product of *MATa* the  $\alpha 2$  product forms a regulatory species called **a1- $\alpha 2$** ; this represses  $\alpha 1$  synthesis as well as haploid-specific genes (Strathern *et al.*, 1981). Diploid homozygotes lacking **a1- $\alpha 2$**  activity can mate - so this product provides a signal to the cell that mating has occurred; the signal turns off expression of genes for mating and turns on the process of sporulation.

### **Sporulation: the Meiotic Cell Cycle**

Sporulation is initiated in **a**/ $\alpha$  diploids by an environmental starvation for nitrogen and glucose, (Esposito and Klapholtz, 1981). It is not clear how starvation is monitored; the ubiquitous molecule cyclic adenosine 3',5'-monophosphate (cAMP) has been proposed to play a role (Matsumoto *et al.*, 1983; Matsumoto, 1985), whereas other reports suggest a different signalling process (Olempska-Beer and Freese, 1987; Cameron *et al.*, 1988). Sporulation proceeds from G1 when cells are of critical size (Dawes and Calvert, 1984; Sudbery *et al.*, 1980) and are heterozygous for the mating type locus *MATa*/*MAT $\alpha$* , exhibiting **a1- $\alpha 2$**  activity (Kassir and Simchen, 1976). Some mitochondrial activity is essential since respiratory-deficient *petite* strains are unable to sporulate (Ephrussi and Hottinguer, 1951).

Once sporulation is initiated, cells go through a sequence of events including: premeiotic DNA replication, recombination, meiosis I and II, and spore formation. Although the genetic control of these events is not completely understood, it has been indicated that cells are committed to meiosis once spindle pole body separation has taken place and that this occurs after







premeiotic DNA synthesis (Simchen, 1974).

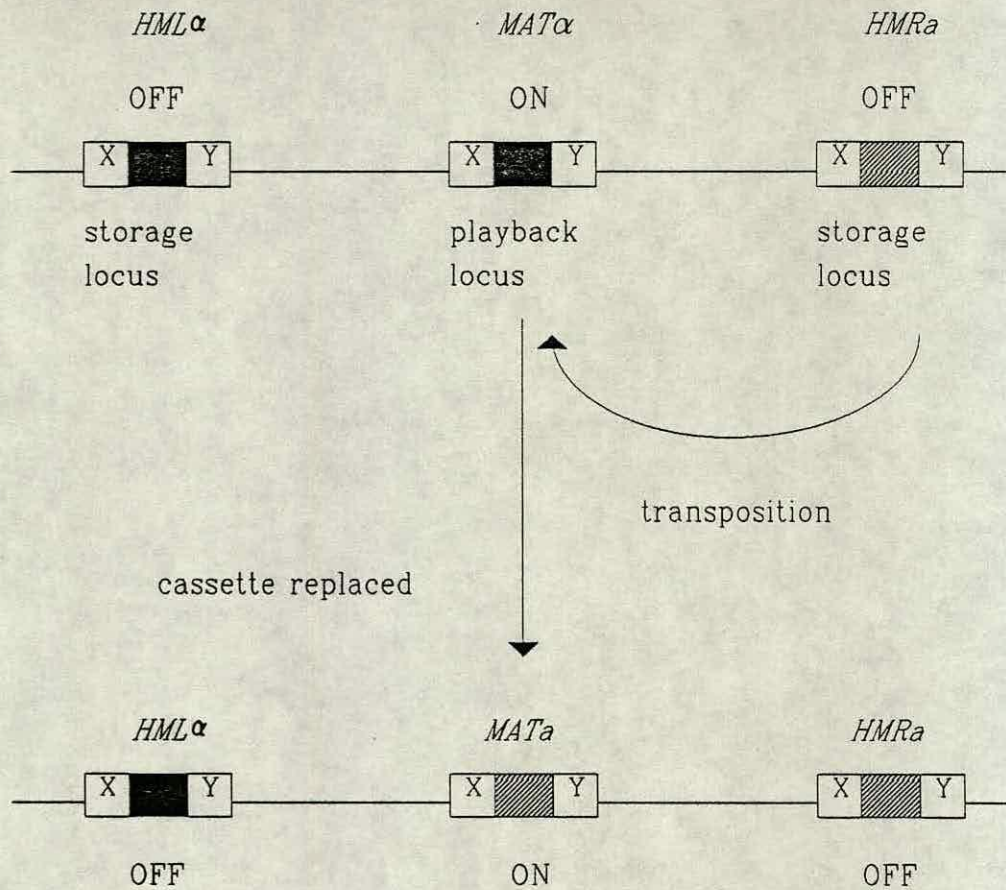
### Homothallic Life Cycle

In a homothallic life cycle, a single haploid cell gives rise to diploid progeny that are capable of undergoing meiosis. In a heterothallic life cycle, diploid progeny are formed only by matings between cells derived from separate spores that have opposite mating types. Homothallic and heterothallic yeasts differ from each other in a single gene, *HO* (Winge and Roberts, 1949). Homothallic strains have the functional version of this gene and cells switch from one mating type to the other as often as every cell division. In *ho* strains, cells switch from one mating type to the other only at low frequency, approximately  $10^{-6}$ .

Figure 2 describes the cassette mechanism for mating-type interconversion. *HO* strains have the ability to change the information at the mating-type locus (Hicks and Herskowitz, 1976; Strathern and Herskowitz, 1979). Silent information becomes activated by a transposition event transferring it from one genomic position, through a recombination event, to another site (Hicks and Herskowitz, 1977; Hicks *et al.*, 1977; Oshima and Takano, 1971). *HML* and *HMR* are loci containing silent copies of this information; *HML $\alpha$*  is the silent version of *MAT $\alpha$*  while *HMR $a$*  is the silent version of *MAT $a$* . A homothallic yeast may switch from *a* to  $\alpha$  by moving *HMR $a$*  to replace *MAT $\alpha$* . The cassettes are kept silent by the action of *SIR* gene products (Buchman *et al.*, 1988; Siliciano and Tatchell, 1986) and movement of the genetic cassette is catalysed by the product of *HO* which codes for a site-specific endonuclease (Strathern *et al.*, 1982; Kostriken and Heffron, 1984; Kostriken *et al.*, 1983); the *HO* protein recognizes a 18 bp site at the mating-type locus (Nickoloff *et al.*, 1986), and makes a double stranded break at this position (Malone and Esposito, 1980). *MAT* is displaced and DNA damage is repaired as the *HMR*







**Figure 2:** The cassette model for homothallic strain mating-type switching in *Saccharomyces cerevisiae*. The cassette *MAT* is expressed but *HML* and *HMR* cassettes are repressed by the *SIR* gene product. Switching to *a* occurs by removing the  $\alpha$  cassette from *MAT* and replacing it by information from *HMRa*. The *Z* and *X* regions are involved in the recombination between cassettes at *HML* or *HMR* loci and the mating-type locus. Mating-type interconversion is initiated by the product of the *HO* gene which codes for an endonuclease that produces a double stranded break at *MAT*. Subsequent repair of the double stranded break leads to a duplicative transposition of information from *HML* or *HMR* to *MAT*.







or *HML* DNA is integrated (Jensen and Herskowitz, 1984; Klar *et al.*, 1982; Strathern and Herskowitz, 1979).

## OVERVIEW OF SPORULATION

Some of the major aims in studying sporulation include defining biochemical and physiological events associated with sporulation, identifying genes and gene-products implicated specifically in sporulation and determining when, in the overall process, these genes act and how they are regulated. During the sporulation process many events occur; these include morphological changes, synthesis and degradation of DNA, RNA, proteins, carbohydrates *etc.*, genetic recombination and chromosome segregation.

Techniques used to study the sporulation process include cytological, morphological and biochemical analysis of normal cells, isolation of mutants with disrupted sporulation events and eventually cloning and characterization of specific genes and gene products which are involved in the sporulation process. Isolation of sporulation-specific genes may be achieved either through the complementation of mutations or by hybridization to mRNAs transcribed preferentially during sporulation.

### Conditions for Sporulation

Diploid cells of the yeast *S. cerevisiae* enter sporulation as part of a starvation response. For sporulation to take place, *a* and  $\alpha$  mating types must be present and the cells must be of a minimum size and in the G1 phase of cell cycle. Cells enter sporulation only under nitrogen limitation and in the presence of a non-fermentable carbon source such as acetate. Similar conditions are needed for spore formation of bacteria, (Fowell, 1975). Moreover, cells must possess functional mitochondria. Glucose and ammonium ions repress sporulation.







**a and  $\alpha$  mating types must be present.** Sporulation requires that cells be a/ $\alpha$  diploids: a/a and  $\alpha/\alpha$  cells are normally unable to sporulate (Roman *et al.*, 1955). Diploids homozygous for the *rme* mutation (Kassir and Simchen, 1976) sporulate regardless of their mating type and studies on these mutants have led to a deeper understanding of mating type control of sporulation (Kassir and Simchen, 1976, Mitchell and Herskowitz, 1986).

**Cell size control.** Calvert and Dawes (1984; 1985) have shown that yeast cells are unable to sporulate until they have reached a volume of about 23  $\mu\text{m}^3$ .

**Sporulation is initiated from the G1 phase of the cell cycle.** It has been shown that the mitotic cell cycle of *S. cerevisiae* includes an initiation point in the G1 phase, called "start". A cell in G1 may initiate the mitotic cell cycle, or sporulation, depending on nutritional conditions (Shilo *et al.*, 1978). "Start" mutants such as *cdc25* and *cdc35* initiate sporulation in rich media, suggesting that the initiation of meiosis includes functions that are shared with "start" of the mitotic cell cycle, as well as functions related to choice between the two processes.

**Some mitochondrial activity is necessary for sporulation to occur.** Certain mitochondrial mutations (*mit*) affecting known respiratory chain components do not prevent sporulation, showing that not all mitochondrial functions are essential for sporulation (Pratje *et al.*, 1979; Hartig and Breitenbach, 1980). However, some mitochondrial functions must be necessary for sporulation since *petite* strains lacking functional mitochondria are unable to sporulate (Ephrussi and Hottinguer, 1951).

**Glucose repression** is thought to work by repression of the tricarboxylic acid (TCA) cycle enzymes required for acetate metabolism (Miyake *et al.*, 1971), or to be due to the known inhibition by glucose of gluconeogenic enzymes







required for the synthesis of storage carbohydrates which accumulate during sporulation (Fraenkel, 1982).

**Nitrogen repression** of sporulation is less well understood and seems to be a complex process (Pinon, 1977; Vezinhet *et al.*, 1979; Cooper, 1982).  $\text{NH}_4^+$  has been shown to block development in other organisms e.g. sporulation in *Bacillus megaterium* (Schaeffer *et al.*, 1965). In yeast,  $\text{NH}_4^+$  inhibits induction of glyoxylate cycle enzymes and adaption to acetate utilization (Gosling and Duggan, 1971) and glycogen degradation (Fonzi *et al.*, 1979). Also affected are RNA and protein synthesis (Durieu-Trautmann and Delavier-Klutchko, 1977; Delavier-Klutchko and Durieu-Trautmann 1978; Delavier-Klutchko *et al.*, 1980) and protein degradation (Croes *et al.*, 1978; Opheim, 1979). Although sporulation is evidently repressed by  $\text{NH}_4^+$ , the mechanism of this effect is unclear since there are conflicting reports on whether it is due directly to ammonium ions or to metabolites synthesized from ammonium ions (Pinon 1977; Dubois *et al.*, 1977; Delavier-Klutchko *et al.*, 1980; Dickinson and Dawes, 1983). The possibility that NADH-dependent glutamate dehydrogenase (GDH) is involved in this regulation was investigated by Newlon and later by Dickinson and Dawes. Newlon reported that nonsense mutations in the gene coding for GDH (*gdhA6*) have no effect on nitrogen sensitivity (Newlon, 1979), whereas Dickinson and Dawes reported that the *gdhA6* mutants were less sensitive to  $\text{NH}_4^+$  than a wild-type strain (Dickinson and Dawes, 1983).

## **MORPHOLOGICAL CHANGES DURING SPORULATION**

Light microscopy, fluorescence microscopy and electron microscopy have all been useful tools in following morphological changes during yeast sporulation. Several groups have analysed morphological events of sporulation (Engels and Croes, 1968; Lynn and Magee, 1970; Moens 1971; Moens and Rapport, 1971; Guth *et al.*, 1972; Beckett *et al.*, 1973).







## PHYSIOLOGICAL AND BIOCHEMICAL CHANGES

Figure 3 indicates the time of occurrence of different events during sporulation.

General DNA and RNA synthesis and degradation have been studied by Croes (1966) and Eposito *et al.* (1969) and changes in levels of storage polymers such as trehalose and glycogen by Kane and Roth (1974) and Lillie and Pringle (1980).

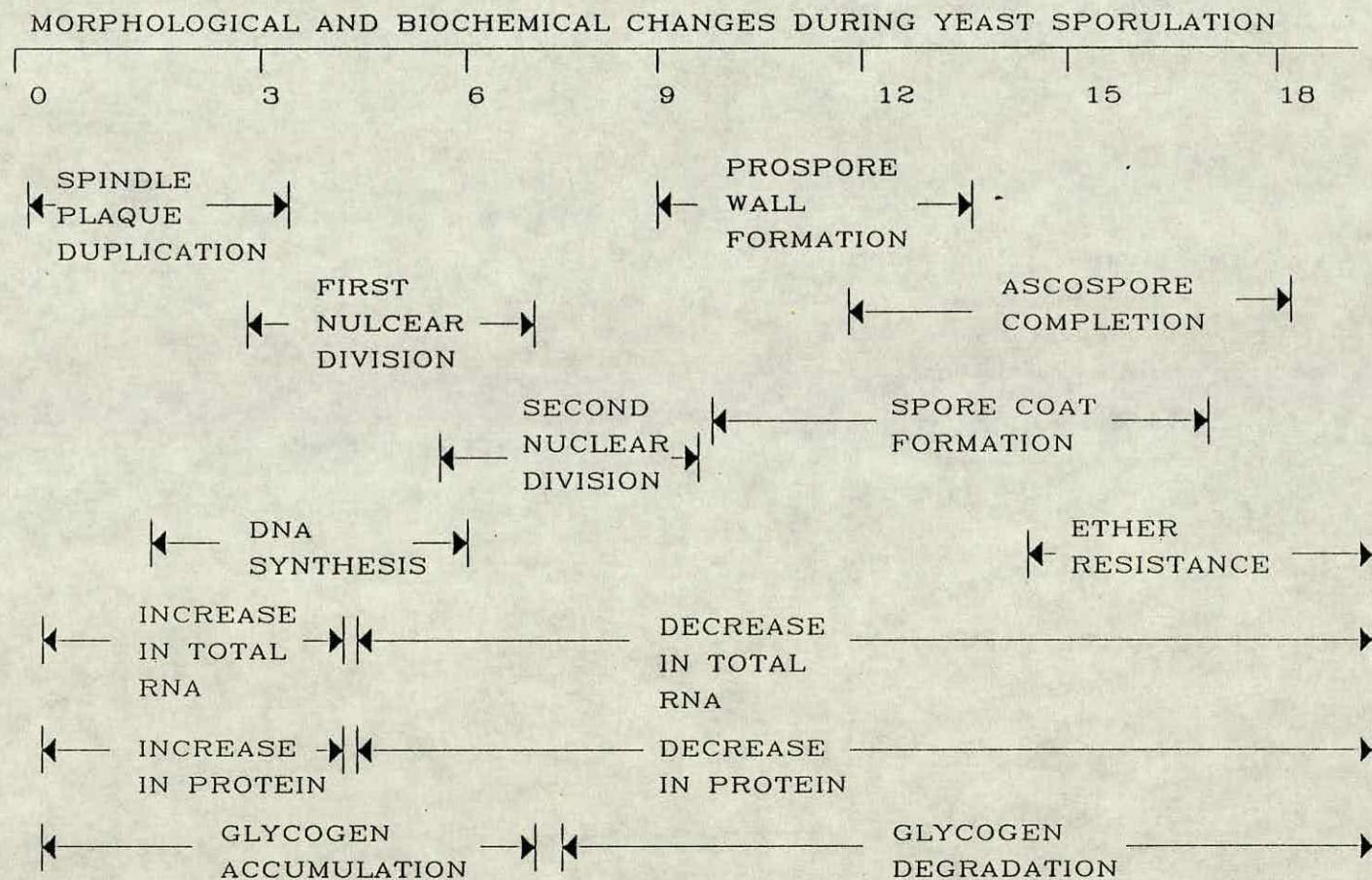
To date, not many of the biochemical processes specific to sporulating cells have been determined in detail, although obvious biochemical events such as the utilization of acetate (Dickinson *et al.*, 1983), premeiotic DNA synthesis (Roth and Lusnak, 1970) and induction of the glyoxylate cycle (Miyake *et al.*, 1971) have been identified as being sporulation specific. It is still difficult to determine sporulation-specific processes precisely, since many of the changes occurring during sporulation are also seen in non-sporulating cells. This is because most of the events seen during sporulation are merely due to the dramatic changes in metabolism when cells are shifted to starvation media and are not events unique or necessary to the sporulation process.

Some of the well known sporulation specific events are discussed.

**Acetate metabolism during sporulation** - A study was carried out to determine more precisely the metabolic changes that accompany sporulation using  $^{13}\text{C}$  NMR to follow acetate metabolism during sporulation (Dickinson *et al.*, 1983). Results showed that there was firstly a very rapid change in the operation of the tricarboxylic acid (TCA) and glyoxylate cycles, such that the intracellular concentration of glutamate increased. From the pattern of labelling of glutamate it was clear that a high proportion of the 2-oxoglutarate produced in the first part of the TCA cycle was converted directly to glutamate. The level of nearly all other amino acids decreased. Trehalose began to be made from acetate via gluconeogenesis 4 h into sporulation and







**Figure 3:** Timing of some morphological and biochemical changes during sporulation in *Saccharomyces cerevisiae*. The times given (h) are approximate.







saturated fatty acids were also made. Vegetatively growing cells did not use acetate for the formation of glutamate.

**Protein synthesis** is necessary for sporulation to occur since inhibitors of protein synthesis stop sporulation (Sando, 1960; Croes, 1967; Esposito *et al.*, 1969; Tingle *et al.*, 1973; Magee and Hopper, 1974).

Since there is no  $\text{NH}_4^+$  in sporulation medium new proteins have to be made using available amino acid sources. Proteinases are involved in this process and mutants with defective proteinase-A are unable to complete sporulation (Betz, 1975).

**Specific protein synthesis.** In order to detect gene products involved directly in sporulation, attempts were made to identify polypeptides produced during sporulation by pulse labelling of sporulating cultures with labelled amino acids, and analysing the patterns of proteins synthesized by polyacrylamide gel electrophoresis (Wright and Dawes, 1979; Wright *et al.*, 1981.). Sporulation-specific mRNA's have been identified by two-dimensional gel electrophoresis (Weir-Thompson and Dawes, 1984; Kurtz and Lindquist, 1984;), and genes that are preferentially expressed during sporulation have been isolated using a differential hybridization screen (Percival-Smith and Segall, 1984; Gottlin-Ninfa and Kaback, 1986; Clancy *et al.*, 1983). Not all genes which are preferentially expressed during sporulation are essential for sporulation; on the other hand some are necessary (Percival-Smith and Segall, 1986; Kao *et al.*, 1989).

**Carbohydrate metabolism.** Carbohydrates are used in spore wall formation and as energy sources for ascus formation and spore germination. A 70% increase in dry weight carbohydrates occurs early in sporulation (Croes, 1967; Esposito *et al.*, 1969) due in the most part to trehalose and glycogen accumulation (Kane and Roth, 1974). Trehalose is synthesized continuously during sporulation reaching a level tenfold higher than that found in vegetative







cells and mutants defective in trehalose do not sporulate (Lillie and Pringle, 1980).

Degradation of glycogen occurs later in sporulation and is thought to be responsible for increased glucose levels (Hopper *et al.*, 1974. Kane and Roth, 1974; Fonzi *et al.*, 1979). Breakdown of glycogen is known to be sporulation specific and the *SGA* gene has been shown to encode an intracellular sporulation-specific glucoamylase in *S. cerevisiae* (Colonna and Magee, 1978; Pretorius *et al* 1986; Pretorius *et al.*, 1986b; Yamashita and Fukui 1985). The induction of *SGA* is controlled by the *MAT* locus (Dranginis, 1989).

## **GENETIC RECOMBINATION AND CHROMOSOME SEGREGATION**

For a review on genetic recombination and chromosome segregation occurring during sporulation see the review article by Esposito and Klapholtz (1981).

## **GENETIC CONTROL OF MEIOSIS AND SPORE FORMATION**

Usually, in the genetic analysis of systems, mutants are selected which disrupt the normal process of events due to mutation of a single gene. Using several different mutants with disrupted functions it is possible to determine the co-ordination of events occurring in the particular system. With the advent of molecular techniques it is now possible to isolate genes by complementation of a particular defect, allowing gene expression and protein function to be analysed in a controlled manner. To study sporulation mutants it is preferable to use homothallic strains because most mutations are recessive and would not be recognized easily in heterothallic diploids. Mutants were sought showing absence of sporulation, reduction in spore number per ascus, reduction in spore viability, absence of intragenic recombination during meiosis, sporulation of mating-type homozygotes or sporulation of glucose grown cells in stationary phase. The methods used to







select and recover sporulation mutants include those of Esposito and Esposito (1975) who selected asporogenous mutants, Hopper and Hall who selected mutations specifically altering mating-type control, and Dawes (1975) who selected mutants which sporulate under condition which normally repress sporulation. It is worth noting, however, that sporulation-defective variants have been found by screening mutants affecting other aspects of cell development: for instance, *cdc* (Simchen, 1974, Shilo *et al.*, 1978), *rad* (Game *et al.*, 1980; Prakash *et al.*, 1980; Malone and Esposito, 1980) and *mat* mutations (MacKay and Manney, 1974; Kassir and Simchen, 1976) all have effects on sporulation. Sporulation-defective mutants have also been revealed by genetic analysis of strains bearing mutations in genes that, *a priori*, would not be expected to be required for sporulation: ochre suppressor mutants, *SUP3*, (Rothstein *et al.*, 1977) and *spe2* mutants, which are lacking S-adenosylmethionine decarboxylase activity (Cohn *et al.*, 1978), are examples. Genes in other, fairly diverse areas are also needed for efficient sporulation to occur. These include loci coding for fatty acid metabolism (Keith, 1969), TCA cycle functions (Ogur, 1965) and several loci required for amino acid metabolism (Wejksnora and Haber, 1974).

There are many levels of control over the sporulation process e.g. the initiation of sporulation, the sequential timing and ordering of gene expression leading to premeiotic DNA replication, recombination, meiosis, and spore formation. With such a wide range of different processes affecting sporulation, the task of determining the precise sequence of events involved in sporulation is not an easy one. The research in this thesis is concentrated on the INITIATION of sporulation. From studies in this area, a clearer picture of events controlling the decision of a cell to sporulate is now forming. Research which has contributed to this understanding, includes the study of sporulation mutants found in the cAMP cascade pathway, mutants involved in mating-type control, guanine auxotrophs, TCA cycle mutants and mutants derepressed for nitrogen repression of sporulation.







## ADENOSINE 3',5'-monophosphate - ROLE IN CONTROL OF SPORULATION

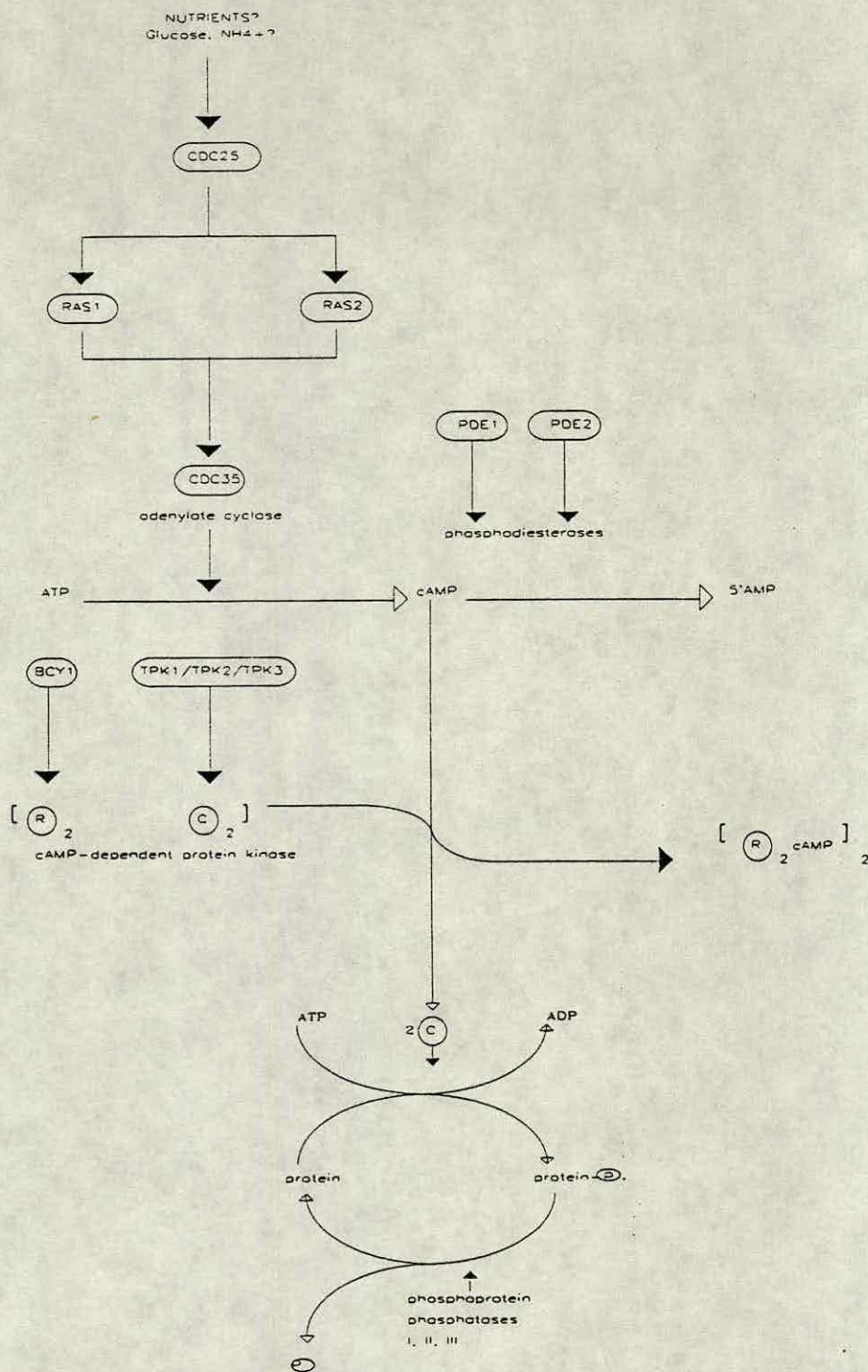
In *S. cerevisiae*, adenosine 3',5'-monophosphate (cAMP) is required for progression of the cell through the division cycle, its effect being mediated by cAMP-dependent protein kinases (Matsumoto *et al.*, 1983). [See Figure 4].

Mutants in the cAMP signal pathway fall into two categories with respect to their effects on sporulation. The first category is found to sporulate on non-fermentable carbon sources such as glycerol even in the presence of a nitrogen source. These mutants characteristically are unable to carry out phosphorylation reactions; this may be due to absence of cAMP synthesis by adenylate cyclase (*cdc25*, *ras2*, *cdc35*), or due to an inactive protein kinase (*cyr2*, *tpk1*, *tpk2*, *tpk3*). The second category of mutants possesses constitutive cAMP-dependent protein kinase activity and cells are sporulation deficient. These mutants may either have high levels of cAMP (*RAS2 val19*, *pde1*, *pde2*) or may have a protein kinase whose activity is independent of cAMP (*bcy1*, overexpression of *TPK1*), [see Table 1]. On the basis of these findings, Matsumoto and his colleagues proposed that initiation of meiosis may require a low level of cAMP or absence of cAMP-dependent protein kinase activity (Matsumoto *et al.*, 1983; Matsumoto *et al.*, 1985). The *CDC25* gene, which has been isolated by complementing *cdc25-1* or *cdc25-5* mutants with wild-type yeast genomic libraries (Camonis *et al.*, 1986; Daniel and Simchen, 1986; Martegani *et al.*, 1986; Lisiewicz *et al.*, 1987; Robinson *et al.*, 1987; Broek *et al.*, 1987), is thought to be responsible for activating *RAS* proteins in response to nutritional signals. *cdc25* mutants have low cAMP levels when arrested at the restrictive temperature (Camonis *et al.*, 1986) and these mutants sporulate in rich growth media (Shilo *et al.*, 1978). The *CDC25* protein appears to have the most upstream function within the cAMP control chain (Robinson *et al.*, 1987; Nikawa *et al.*, 1987) and is absolutely required in wild-type cells for the activation of the *RAS*/adenylate cyclase









**Figure 4:** Scheme for cAMP metabolism and mutations in *Saccharomyces cerevisiae*. Dark arrows indicate activation. Functions of the genes *CDC25*, *CDC35*, *RAS1*, *RAS2*, *BCY*, *TPK1/2/3* are described in Table 1. The *PDE1* and *PDE2* genes are described by Wilson and Tatchell, (1988) and Sass et al., (1986) respectively.







pathway by stimulating the formation of the activating *RAS*-GTP complex (Broek *et al.*, 1987). *CDC25* is thought to encode a protein which acts as a GDP-GTP exchange factor regulating the activity of the *RAS2* gene product (Camonis *et al.*, 1988). The *CDC25* protein is essential for viability and mitotic growth in the presence of the *BCY*-encoded cAMP receptor protein (Toda *et al.*, 1987) and wild-type *RAS* proteins (Toda *et al.*, 1985; Marshall *et al.*, 1987). Three functionally different domains have been identified within the *CDC25* gene (Munder, 1988): one region is essential for viability (mitotic growth on glucose media), a second region is required for sporulation, but not for mitotic growth, and a third region is required for sporulation and gluconeogenic growth, but not for growth in glucose media. Induced mutations in the *CDC25* gene could produce both "hypersporulation" and asporogenous phenotypes leading Munder and his colleagues to propose that sporulation may require cAMP-dependent as well as cAMP-independent functions.

***RAS2* gene and sporulation.** Two gene homologues of the proto-oncogene *ras* (Barbacid, 1987) have been shown to be necessary for cAMP production: *RAS1* and *RAS2* (DeFeo-Jones *et al.*, 1983; Powers *et al.*, 1984). Genetic and biochemical evidence indicates that yeast *RAS* products stimulate adenylate cyclase activity and that the glucose-induced cAMP signal in *S. cerevisiae* is mediated by the *RAS* products, (Mbonyi *et al.*, 1988) which are GTP- and GDP-binding proteins with GTPase activity (Temeles *et al.*, 1984; Tamanoi, 1984; Temeles, 1985). The loss of *RAS2* alone results in several defects, including an inability to grow on non-fermentable carbon sources such as glycerol and ethanol, a hyperaccumulation of the storage carbohydrates glycogen and trehalose and a lack of nutrient repression of sporulation (Fraenkel, 1985). Surprisingly, mutations in *RAS1* have none of these phenotypes (Tatchell *et al.*, 1985).

The *bcy1* mutants bypass the requirement for *RAS* in the cAMP pathway, since they have decreased levels of the regulatory subunit of the cAMP-dependent protein kinase, thereby allowing the protein kinase to function in







the absence of cAMP. The *BCY* gene codes for the regulatory subunit of the cAMP-dependent protein kinase (Yamano *et al.*, 1987). The *bcy1* mutations have a recessive asporogenous phenotype, decreased viability under nitrogen starvation, and accumulate a brown pigment when cultured for long periods. The *RAS2<sup>val19</sup>* mutation (Kataoka *et al.*, 1985; Sigal *et al.*, 1986), which results in hyperactivation of *RAS*, gives the same phenotype as *bcy1*, but an extragenic suppressor of the *cdc25* mutation, *RAS2<sup>ile152</sup>* (Crechet *et al.*, 1990) which results in a reduced capacity of the *RAS* protein to bind guanosine nucleotides (Crechet, Camonis *et al.*, in press) gives a much more normal phenotype.

*TPK1*, *TPK2*, *TPK3* are three genes which code for catalytic subunits of the *S. cerevisiae* cAMP-dependent protein kinase (Toda *et al.*, 1987). Gene disruption experiments demonstrated that no two of the three genes are essential by themselves but at least one *TPK* gene is required for a cell to grow normally. Mutant *TPK* genes which appear to encode functionally attenuated catalytic subunits of the cAMP protein kinase (*tpk<sup>w</sup>*) have been isolated (Toda *et al.*, 1987). *tpk<sup>w</sup>* mutations suppress all of the *bcy1* defects and allow yeast strains to respond appropriately to nutrient conditions even in the absence of *CDC25*, both *RAS* genes or *CYR1*, which are the components of the cAMP-generating machinery (Cameron *et al.*, 1988). These results suggest that cAMP-independent mechanisms must exist for regulating glycogen accumulation and sporulation in *S. cerevisiae*.

## **cAMP-INDEPENDENT CONTROL OF SPORULATION**

Guanine auxotrophs initiate meiosis and sporulation of *S. cerevisiae* through guanine deprivation (Bautz-Freese *et al.*, 1984). Results show that sporulation also takes place in media rich in nitrogen in which only carbon or sulphur are limited (Freese *et al.*, 1982, 1984). Under these conditions as well as under nitrogen source starvation, GTP and GDP levels decrease while changes in







other nucleoside tri- and diphosphates show no correlation with sporulation (Olempska-Beer and Freese, 1987). Freese and his colleagues studied Matsumoto's hypothesis that sporulation was initiated by low cAMP levels (Matsumoto, 1983) by studying the effects of guanine starvation on intracellular cAMP and sporulation in a guanine auxotroph. They tested yeast strains on media containing yeast nitrogen base and potassium acetate but differing with respect to additions of adenine, guanine and 3-isobutyl-1-methylxanthine (IBMX; a known inhibitor of cAMP phosphodiesterase). Results showed that the combination of adenine and IBMX caused guanine deprivation (uptake of guanine was inhibited by adenine), increased the intracellular cAMP concentration slightly above the level in vegetatively growing cells, and allowed excellent sporulation. Throughout sporulation, cAMP was excreted into the medium. In brief, their results showed that *S. cerevisiae* can enter meiosis and sporulate while the intracellular concentration of cAMP remains essentially constant. These results are in disagreement with Matsumoto's proposal that initiation of sporulation requires a decrease of cAMP.

In conclusion, from research on the cAMP signal pathway it appears that cAMP may play a role in the initiation of sporulation (Matsumoto, 1985). However, it has also been shown that cAMP on its own does not tell the whole story concerning sporulation control and indications that cAMP-independent controls are also present (Cameron *et al.*, 1988; Munder *et al.*, 1988) are being taken seriously. This theory is further supported by studies on RAS-related GTP-binding proteins encoded by the genes *GPA1* (Miyajima *et al.*, 1987) and *GPA2* (Nakafuku *et al.*, 1988), guanine auxotrophs (Olempska-Beer and Freese, 1984) and the *SPD* genes (Dawes and Calvert, 1984).







## MATING-TYPE CONTROL OF SPORULATION - THE INVOLVEMENT OF *IME/RME* GENES

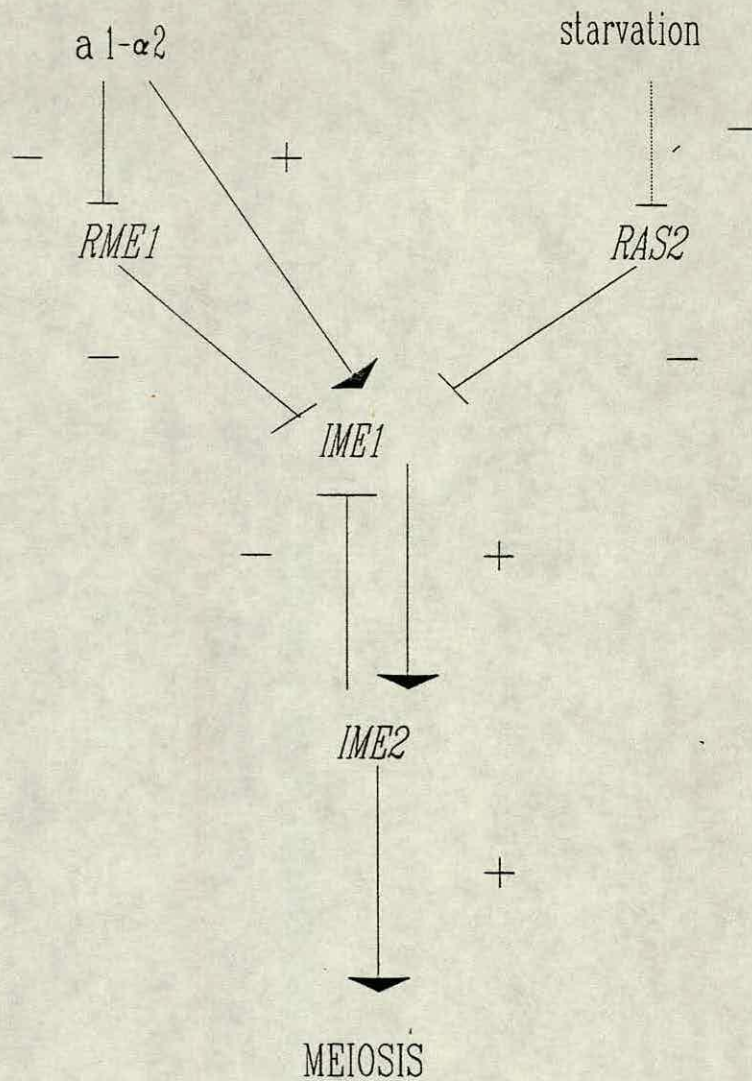
*RME1* (Regulator of **ME**iosis). In trying to determine how the *MAT* locus controls the ability of cells to sporulate, it was found that cells homozygous for the *rme* mutation sporulate, regardless of their mating type (Kassir and Simchen, 1976). The *RME1* gene is thought to be a member of the haploid-specific gene family whose expression is switched off in a diploid cell by the  $a1-\alpha2$  activity (Mitchell and Herskowitz, 1986). In other words, the presence of *RME* is thought to block sporulation, while its absence allows sporulation, bypassing the need for mating-type control. Inactivation of the *RME* product does NOT relieve nutritional control; likewise, nutritional control bypass mutations such as *cyr1-1* do not abolish the *MAT* requirement.

*IME1* (Inducer of **ME**iosis) in high copy number enables *MAT* insufficient strains to undergo meiosis. *IME1* is an inducer of meiosis, whose transcription is repressed by *RME1*, glucose and nitrogen (Kassir *et al.*, 1988). *IME* on a multicopy plasmid vector confers a phenotype similar to the mutations *cdc25*, *cdc35*, *ras2* and *spd*'s: i.e. sporulation on rich media. After starvation, the level of the *IME1* transcript increases only in the absence of the *RME1* product. Kassir and her colleagues have suggested that *IME* is the point at which the two controls of meiosis - environment and mating-type merge. Granot and colleagues looked at upstream sequences of the *IME1* gene and their effect on regulation of meiosis (Granot *et al.*, 1989). Both positive and negative regulatory sites were found; upstream activating sites enhance the expression of *IME1* while other sites seem to be responsible for negative regulation of *IME* transcription by the *RME1* product. It also seems likely that the long upstream region of *IME1* contains negative regulatory sites which mediate nutritional control of *IME1*.









**Figure 5:** Transcriptional cascade that governs entry into meiosis, a model proposed by Smith and Mitchell (1989). The *IME2* product is necessary for meiotic recombination and spore formation. The *IME1* product is required for *IME2* expression. The *IME2* product is a negative regulator of *IME1*, acting after prolonged starvation. The *RME1* product is a negative regulator of *IME1* expression. The *MAT* products  $\alpha 1$  and  $\alpha 2$  block *RME1* expression.  $\alpha 1$  and  $\alpha 2$  are thought to stimulate *IME1* expression through a *RME1*-independent mechanism. *RAS2* blocks *IME1* expression in rich medium. It is possible that starvation may inhibit *RAS2* activity to permit expression of *IME1*.







*IME2*, which was cloned by Smith and Mitchell (1989), has properties similar to *IME1*. Its expression is activated by the same signals that activate meiosis: starvation and the *MAT $\alpha$ 1* and *MAT $\alpha$ 2* products. *IME1* was found to be required for *IME2* expression, and a multicopy *IME2* plasmid permits meiosis in an *ime1* deletion mutant. From this information it is proposed that the *IME1* product stimulates meiosis through activation of *IME2* expression since the *ime1* null mutation blocks accumulation of *IME2* mRNA. The *ras2* mutation which causes diminished adenylate cyclase activity bypasses nutritional inhibition of *IME1* and *IME2* expression. It is therefore postulated that regulators of *IME1* expression may be targets of the cAMP dependent protein kinases.

Smith and Mitchell have proposed that a transcriptional cascade involving *RME1*, *IME1*, *IME2*, and others, controls entry into meiosis and that the cAMP pathway converges on this cascade pathway, perhaps at *IME1* (Figure 5).

#### **SPD GENES - *spd* MUTANTS SHOW THE STRONGEST EFFECTS OF SPORULATION DEREPRESSION**

**Isolation of *spd* mutants.** Asporogenous mutants have been used to study the genetic control of sporulation in *S. cerevisiae* (Esposito and Esposito, 1969; Esposito *et al.*, 1972; Tsuboi, 1983), however, Dawes reasoned that in such developmentally blocked mutants, it would prove difficult to define the precise lesion of a mutation and its place of control in a sequence of reactions. In an alternative approach to study sporulation control, he looked for DEREPRESSED mutants *i.e.* those which undergo development in conditions in which the wild-type does not (Dawes, 1975). Such mutants were isolated by germinating mutagenized ascospores of a homothallic yeast strain grown to stationary phase and treated with ether to selectively kill vegetative cells (Dawes and Hardie, 1974). This treatment was repeated several times and derepressed mutants were recovered by plating survivors of the final ether treatment on glucose medium. Mutants selected, sporulated at high



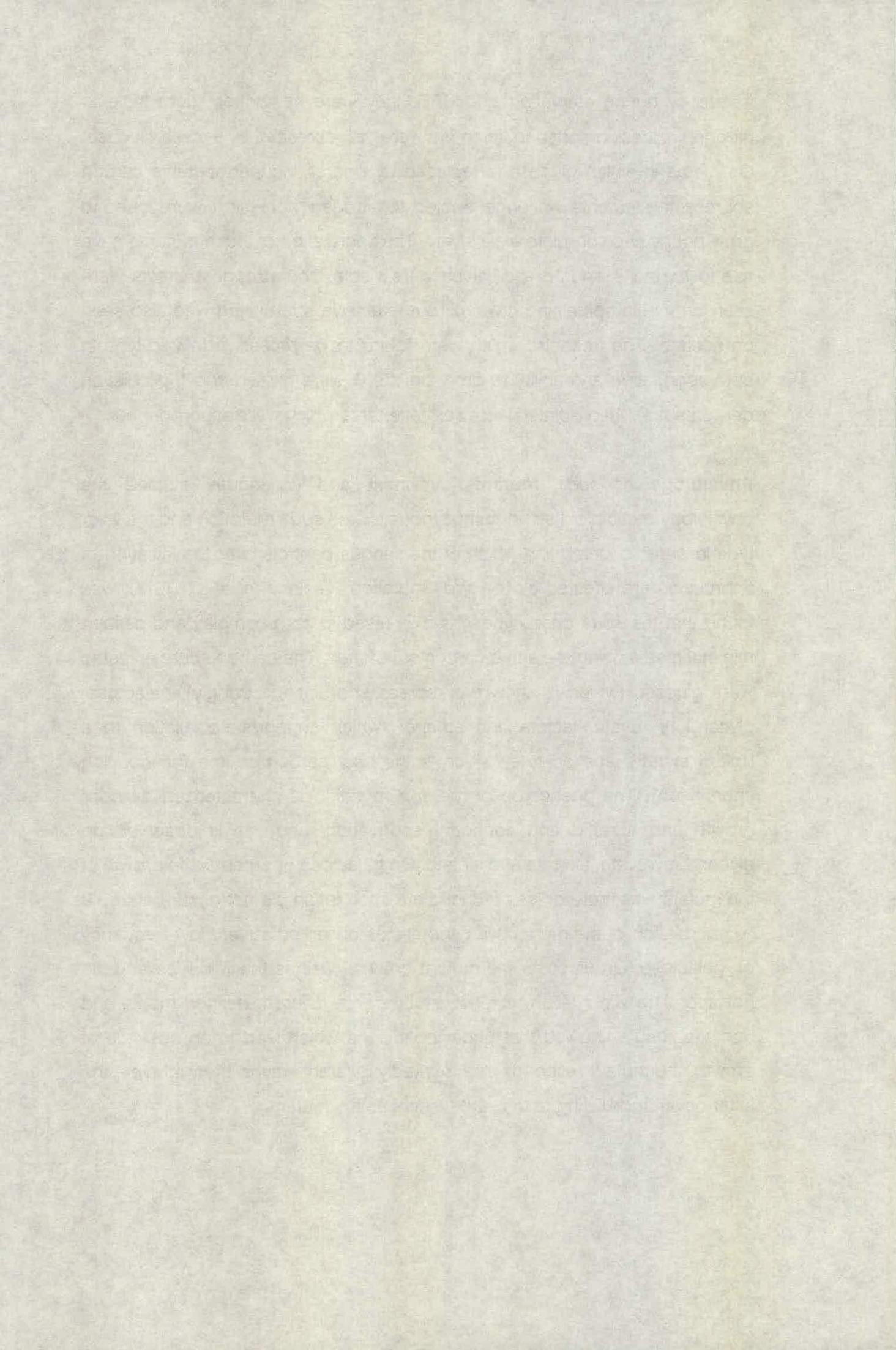




frequency during starvation on YEPD; they were no longer susceptible to nitrogen repression of sporulation but were still repressed by excess glucose. On media in which glucose is replaced by poorer, non-fermentable carbon sources, the mutants, no longer subject to nitrogen repression, were found to grow poorly and sporulate extensively. The sporulation of these mutants gave rise to normal 4 spored asci which were viable. The strongest effects were seen on media containing glycerol, but extensive sporulation was also seen on acetate. The mutants, which were found to be recessive to wild-type in both sporulation and ability to grow on YEPG were named *spd* (sporulation derepressed). Also isolated were *spd* revertants which were asporogenous.

**Physiology of *spd1* mutants.** Vezinhet and colleagues studied the physiology of a diploid strain homozygous for the *spd1* mutation and the wild-type in order to determine which of the various controls over the initiation of sporulation are affected by the *spd1* mutation (Vezinhet *et al.*, 1979). It was found that the *spd1* phenotype was expressed in both complex and defined minimal media using the same carbon substrates. The carbon sources tested were glucose (which is known to repress sporulation strongly), galactose, glycerol, pyruvate, lactate and ethanol (which supports sporulation to a limited extent), and acetate (which is the best carbon source for inducing sporulation). The phenotype of the *spd* mutation is characterized by poor growth and a rapid and abundant sporulation, and this is observed on glycerol, pyruvate, acetate and lactate. On galactose or ethanol, the growth of the mutant was more or less reduced but sporulation did not occur before 48 h. Sporulation of the mutant was sometimes observed at very low frequency on galactose. On glucose, the mutant grew as well as the wild-type and did not sporulate within 48 h. In brief, studies with different defined media and complex media showed that under conditions which lead to an absence of growth, the mutant responds in a markedly different way to the wild type, the latter never sporulating to the same extent as the mutant.







**Metabolism of glycerol.** Since the *spd* phenotype is very clearly expressed in glycerol, Vezinhet and colleagues compared the metabolism of wild-type and *spd1* mutants on this carbon source to verify whether the mutant was affected in glycerol uptake (Vezinhet *et al.*, 1979). Uptake of [ $^{14}\text{C}$ ]-glycerol was studied over a short period; the rate of incorporation of the isotope was similar in both the wild-type and the mutant. The activities of two enzymes - glycerol kinase and glycerol-3-phosphate dehydrogenase - which are involved directly in glycerol catabolism, were measured but no significant differences were found between the *spd1* and wild-type levels. From these results, it was concluded that the *spd1* mutants are not affected in the uptake of glycerol or the synthesis of the enzymes concerned with its entry into general metabolic pathways but that the mutants are probably affected in a central metabolic function which is essential for the utilization of all of the substrates on which the *spd1* phenotype is expressed.

**Haploid phenotype.** The *spd* mutation is also expressed in haploids - these mutants become arrested in the G1 phase of cell cycle at, or before the execution point for the *cdc28* mutation (Vezinhet *et al.*, 1979).

**$\text{NH}_4^+$  repression of sporulation in *spd1* diploids.** Vezinhet and colleagues showed that, under certain conditions,  $\text{NH}_4^+$  does not repress sporulation of *spd1* mutants (Vezinhet *et al.*, 1979). On buffered potassium acetate, and potassium acetate containing vitamins and essential ions (YNB-N) the difference between the wild-type and the mutant is very marked - sporulation of the mutant being almost completely insensitive to  $\text{NH}_4^+$ .

An *spd1* mutant was tested for its ability to repress synthesis of three enzymes normally repressed by nitrogen - arginase,  $\text{NAD}^+$ -dependent glutamate dehydrogenase and glutamine synthetase (Middlehoven, 1970; Ferguson and Sims, 1971; Prival *et al.*, 1973; Dubois *et al.*, 1973). The mutant strain was found to repress these enzymes when  $\text{NH}_4^+$  was present, as does the wild-type. However, the basal level of the  $\text{NAD}^+$ -dependent







glutamate dehydrogenase in the *spd1* mutants was found to be higher (Vezinhet *et al.*, 1979).

$\text{NH}_4^+$  has been shown to act at 2 different stages during sporulation; one early in initiation and the other concerned with the organization and delimitation of mature spores (Dickinson and Dawes, 1983). A strain homozygous for the *spd1* gene showed a similar  $\text{NH}_4^+$  sensitivity response to the wild-type with respect to the formation of complete asci, however the strains differed in their relative abilities to initiate sporulation, the *spd1* mutant being less sensitive to  $\text{NH}_4^+$  repression of sporulation. In contrast with the results of Newlon (1979), Dickinson and Dawes (1983) showed that mutants homozygous for the *gdhA6* mutation (the structural gene for the anabolic  $\text{NADP}^+$ -dependent glutamate dehydrogenase) also resulted in a loss of sensitivity to  $\text{NH}_4^+$  inhibition of initiation of sporulation as found in *spd1* mutants. In view of these findings, Dickinson and Dawes suggested that regulation of  $\text{NADP}^+$ -dependent glutamate dehydrogenase may be altered in *spd1* mutants.

**Genetic characterization of *spd* mutants.** *spd* mutants were found to belong to three independent linkage groups - these groups being represented by *spd1*, *spd3* and *spd4* mutations. The *spd1* mutation was found to be centromere linked, and was mapped to the left hand side of chromosome XV near *SUP3* (Dawes and Calvert, 1984). The mutations were found to be the result of single recessive nuclear mutations, although some mutations were not completely recessive since heterozygous diploids often sporulated to a slightly greater extent than the wild-type on YEPG medium. None of these mutations were linked to the cell cycle mutations *cdc25*, *cdc35*, *cdc28*. Neither were the *spd* mutations allelic to any of the mutations in the cAMP signal pathway which were tested e.g. *ras1*, *ras2*, *cdc25*, *cdc35* (Janice Doull - personal communication).







**spo 50, 51 and 53 mutants** - spontaneous suppressors of *spd*.

*spd* mutations are unstable since they exhibit a high frequency of reversion with respect to ability to grow on glycerol e.g. for *spd1* there was one revertant for  $3 \times 10^4$  cells of an early stationary phase culture grown on YEPD (Dawes and Calvert, 1984). These were not real revertants, however, but were shown to result from extragenic suppressors corresponding to three unlinked loci, designated *spo50*, *spo51* and *spo53*. They were found to be recessive to wild type with respect to sporulation but dominant with regard to suppression of *spd1* poor growth on non-fermentable carbon sources (Calvert and Dawes, 1984b). The mutations *spo50*, *spo51*, *spo53* all suppressed both *spd1* and *spd3* mutations, implying that all of these mutations affect a common pathway in the cell. Other characteristics of *spo* mutants include a loss of viability when stored, abnormal morphology producing long mycelium-like processes under starvation conditions and the acquisition of a distinct brown colour after prolonged incubation on YEPD.

## **THE *spd* MUTANTS - LINKS WITH GLUCONEOGENESIS AND TRICARBOXYLIC ACID CYCLE OPERATION**

The tricarboxylic acid (TCA) cycle supplies intermediates for a large number of different biosynthetic pathways and is also the major route for the oxidative degradation of carbohydrates, fats and amino acids (see *The Molecular Biology of the Yeast Saccharomyces cerevisiae: Metabolism and Gene Expression*; p.1-37; Strathern, Jones and Broach, 1982). TCA cycle mutants have been shown to affect sporulation (Dickinson *et al.*, 1986) and also growth on non-fermentable carbon sources (Subik *et al.*, 1972; Dickinson *et al.*, 1986; Repetto and Tzagoloff, 1989). Furthermore, isocitrate lyase defective mutants do not sporulate (Miclo and Guesdon, 1980) indicating that a functional glyoxylate cycle is also necessary for sporulation. Biochemical analysis of *spd1* mutants has shown that they may be affected in the regulation of the TCA cycle since mutants accumulate L-glutamate and







several metabolites involving glutamate or 2-oxoglutarate in their metabolism (glutathione, glycyl-proline and saccharopine) while activity of 2-oxoglutarate dehydrogenase is lower than in wild-type strains (Dickinson *et al.*, 1985).

**Role of Gluconeogenesis in Sporulation.** Dickinson used mutants in gluconeogenesis and the pentose phosphate pathway to study requirements for sporulation (1986). Homozygous mutants for the *spd1* gene resulted in sporulation of strains homozygous for glycolysis or gluconeogenesis asporogenous mutants. Dickinson proposed that a possible explanation for this effect relies on an understanding of the regulation of carbon metabolism affected by the amount of  $\text{NH}_4^+$  available to the cell. Cells starved of  $\text{NH}_4^+$  accumulate glycogen and trehalose (Trevelyan and Harrison, 1956), whereas available  $\text{NH}_4^+$  stimulates glycolysis (Saita and Slaughter, 1984).  $\text{NH}_4^+$  is therefore antagonistic towards gluconeogenesis but stimulates glycolysis. In *spd* mutants, a large amount of metabolized carbon is diverted out of the TCA cycle to glutamate and glutamate-derived metabolites (Dickinson *et al.*, 1985) resulting in an excess of glutamate and a removal of  $\text{NH}_4^+$  from the cytoplasm. Dickinson suggests that low levels of available  $\text{NH}_4^+$  may be responsible for an increase in activity of the gluconeogenic pathway and a subsequent increase in ability to sporulate (Dickinson and Williams, 1986).







**Table 1: Mutations affecting sporulation initiation.**

MUTATION	GENE FUNCTION	COMMENTS	REFERENCE
<i>cdc35</i>	structural gene for adenylate cyclase	sporulate on YEPG medium - suppressed by <i>bcy1</i>	Matsumoto <i>et al.</i> , (1984) Boutelet <i>et al.</i> , (1985) Yamano <i>et al.</i> , (1987)
<i>cdc25</i>	RAS protein activator	sporulate on YEPG medium - suppressed by <i>SCDC25</i> , <i>RAS<sup>ileu152</sup></i> , <i>RAS<sup>val19</sup></i> ; also <i>SCH9</i> on multi-copy vector	Robinson <i>et al.</i> , (1987) Powers <i>et al.</i> , (1989) Kataoka <i>et al.</i> , (1985) Camonis and Jacquet, (1988) Toda <i>et al.</i> , (1989)
<i>ras2</i>	GTP/GDP-binding protein	sporulate on YEPG medium - suppressed by <i>RAS<sup>val19</sup></i> , also <i>SCH9</i> on multi-copy vector	DeFeo-Jones <i>et al.</i> , (1983) Powers <i>et al.</i> , (1984)
<i>tpk1/2/3</i>	catalytic subunits of cAMP-dependent protein kinase	<i>tpk<sup>W</sup></i> mutants suppress <i>bcy1</i> and allow strains to respond appropriately to nutrient conditions even in the presence of <i>ras1</i> , <i>ras2</i> , <i>cdc25</i> or <i>cdc35</i> mutations	Toda <i>et al.</i> , (1987) Cannon and Tatchell, (1987) Lisiewicz <i>et al.</i> , (1987) Cameron <i>et al.</i> , (1988)
<i>spd1/3/4</i>	function unknown	sporulate on YEPG medium - are insensitive to repression of sporulation by nitrogen - suppressed by <i>spo/51/53</i> ; also by the <i>SCH9</i> gene product on multi-copy vector	Dawes <i>et al.</i> , (1984) Vezinhet <i>et al.</i> , (1979) Calvert and Dawes, (1984)
<i>gua</i>	implicated in guanine synthesis pathway	sporulation independent of cAMP concentration	Bautz Freese <i>et al.</i> , (1984)
<i>rme1</i>	regulator of meiosis	sporulation independent of mating-type	Mitchell and Herskowitz, (1986)
<i>ime1</i>	inducer of meiosis	sporulation inhibited in mutant - gene in multi-copy induces sporulation on YEPG	Kassir <i>et al.</i> , (1988)
<i>ime2</i>	inducer of meiosis	stimulates meiosis through activation of <i>IME2</i> expression	Smith and Mitchell, (1989)
<i>lpd</i>	structural gene for lipoamide dehydrogenase	inhibits sporulation in <i>LPD/LPD</i> diploid but heterozygote <i>lpd/LPD</i> sporulates more than <i>LPD/LPD</i> . Structural gene suppresses <i>spd</i> 's with respect to growth on YEPG	Dickinson <i>et al.</i> , (1986) Roy and Dawes, (1987) Ross <i>et al.</i> , (1988)







## AIMS OF RESEARCH

An important step in characterizing further the nature of the *spd1* mutation involves cloning and sequencing of the gene in order to determine when it is expressed and to determine its function. Dr. Janice Doull attempted to clone the *SPD1* gene by complementation of the defect in the *spd1* mutant. She isolated a plasmid, pJLD1, containing a 4.5 kb DNA fragment which suppressed an *spd1* mutant's inability to grow on the non-fermentable carbon source glycerol.

The aims of the work described in this thesis were to characterize the sequence contained in the pJLD1 plasmid by determining whether:

- i) this plasmid contains the *SPD1* gene or an extragenic suppressor of *SPD1* and
- ii) whether or not the sequence suppresses the hypersporulation phenotype of *spd1* mutants.

Since, early on, the pJLD1 plasmid was found to contain a gene other than *SPD1*, work was carried out to characterize this sequence through *in vivo* gene disruption experiments and sequence analysis.

Homozygous diploid *spd1* mutants have a similar phenotype to some of the cell cycle mutants including *cdc25*, *ras2* and *cdc35*, sporulating in rich media. The *CDC25*, *RAS2* and *CDC35* genes all have known functions in the cAMP signal pathway whereas the *SPD1* gene has been shown not to be allelic to any of these genes. A further aim of the work presented in this thesis is to determine more precisely the relationship between the *spd1* mutations and the cAMP pathway.







## METHODS AND MATERIALS







**Table 2:** Genotypes of important strains used**1 *Saccharomyces cerevisiae***

STRAIN	GENOTYPE	SOURCE
S41	<u>MATa HO arg4-1 cyh-1</u> MATa HO arg4-1 cyh-1	H.O Halverson
59.4A	<u>MATa HO arg4-1 cyh1 spd1-1</u> MATa HO arg4-1 cyh1 spd1-1	I. W. Dawes
SF 747.19D	MATa ho leu2-3,112 his4 ura3-52 gal2 MEL4	G. R. Reid
W303	<u>MATa ho his11-15 trp1-1 ade2-1 leu2-3,112 ura3-1 can1-100</u> MATa ho his11-15 trp1-1 ade2-1 leu2-3,112 ura3-1 can1-100	M. Jacquet
410:2C	<u>MATa HO his11-15 leu2-3,112 ura3-1 spd1-1</u> MATa HO his11-15 leu2-3,112 ura3-1 spd1-1	I. W. Dawes
410:3C	<u>MATa HO his4 leu2-3,112 spd1-1</u> MATa HO his4 leu2-3,112 spd1-1	I. W. Dawes
309	<u>MATa HO ura1 spd3</u> MATa HO ura1 spd3	I. W. Dawes
310	<u>MATa HO ura1 spd4-1</u> MATa HO ura1 spd4-1	I. W. Dawes
417	<u>MATa HO arg4-1 leu2-3,112 ura- spd4-1</u> MATa HO arg4-1 leu2-3,112 ura- spd4-1	I. W. Dawes
391	<u>MATa HO leu2-3,112 ura- spd3</u> MATa HO leu2-3,112 ura- spd3	I. W. Dawes
OL97.1.11B	MATa ho cdc25-5 ura3 leu2 his4	M. Jacquet
OL97.1.4D	MATa ho cdc25-5 ura3 leu2 ade1 arg4	M. Jacquet
DOL97	<u>MATa ho cdc25-5 ura3 leu2</u> MATa ho cdc25-5 ura3 leu2	M. Faure

**2 *Escherichia coli***

DH1	F <sup>-</sup> recA1 endA1 gyrA96 thi1 hsdR17 ( <i>r<sub>k</sub><sup>-</sup></i> , <i>m<sub>k</sub><sup>-</sup></i> ) supE44	D. Hanahan
CM5a	supE44 ΔlacU169 (lacZΔM15) chsdR17 recA <sub>1</sub> endA <sub>1</sub> gyrA96 thi-1 relA <sub>1</sub> F' [Tet <sup>r</sup> ]	J. Camonis
159	uvrA gal <sup>-</sup> str <sup>r</sup> sup <sup>0</sup> <i>r<sub>k</sub><sup>-</sup></i>	M. Stark
5K	F <sup>-</sup> thi <sup>-</sup> thr-1 leuB6 lacY1 tonA21 supE44 <i>r<sub>k</sub><sup>-</sup></i> <i>m<sub>k</sub><sup>+</sup></i>	M. Stark







## PLASMIDS AND OTHER DNA

**YEp352:** pUC18+ *URA3* + 2 $\mu$  (Hill, 1986). **YEp13:** pBR322 + *LEU2* + 2 $\mu$  (Broach *et al.*, 1979). **YCp50:** pBR322 + *URA3* + *CEN4* (Rose *et al.*, 1987). M13mp18 and M13mp19: (Yannisch-Perron *et al.*, 1985). **YIp352:** constructed from YEp352 by removing the 2 $\mu$  DNA (Hill, 1986). **pCS19:** pBR322 + *URA3* + *CEN4* (kindly donated by J. Thevelein). **PKS/PSK:** pUC19 + *lacZ* + multiple cloning site (STRATAGENE). **pJLD1:** YEp13 + a 4.8 kb *Sau3A* insert (at the *Bam*HI site) which contains the *SSPD1* gene. (Janice Doull). **pARC1:** YCp50 + a *Bam*HI/*Hind*III 4.8 kb insert containing the *SSPD1* gene. **pAR2:** derived from pJLD1 by deleting the 3.0 kb *Pvu*II/*Pvu*II sequence. **pAR3:** derived from pJLD1 by deleting the 2.8 kb *Pvu*II/*Pvu*II sequence. **pEI6:** derived from pJLD1 by deleting all yeast sequences (except the 4.8 kb *SSPD1* sequence) by *Eco*RI digestion and religation (J. Doull). **pJIVI:** derived from pEI6 by insertion of the *URA3* gene into the *Hind*III site of pEI6 (J. Doull). **pDIVI:** derived from pJIVI by inserting the *URA3* gene into the *Bgl*II site within the *SSPD1* sequence and forming a linear sequence containing the disrupted *SSPD1* sequence by cutting with *Pvu*II. **pSCAR:** pCS19 + a 15 kb *Sau3A* sequence (*SSPD2*) inserted into the *Bcl*I site. **YIpS2:** a 2.4 kb *Pst*I/*Bam*HI fragment from within the *SSPD2* sequence inserted into the multiple cloning site of the integration vector YIp352. **pRG1:** contains the C-terminal part of the *SCD25* gene in the YEp352 vector (Faten Damak). **pRG3:** contains the entire *SCD25* gene in the YEp352 vector (Faten Damak). **pRFC:** contains the *RAS2* gene in YCp50 (Jacques Camonis). **pRYC:** YCp50 + *RAS2<sup>ile152</sup>* (Jacques Camonis). **pHF:** YEp352 + *RAS2* (J. Camonis). **pHY:** YEp352 + *RAS2<sup>ile152</sup>*. **pCB23:** YCp50 + *TPK1* (J. Camonis). **pSL18:** YEp13 + *TPK1* (Simchen). **pL25.6.1:** pBM272 + *CDC25* (Hervé Garreau *et al.*, 1990).

**Gene Banks.** A YEp13-based gene bank kindly donated by K. Nasmyth was used to isolate the *SSPD1* sequence.







A pCS19-based gene bank kindly donated by J. Thevelein was used to isolate the *SSPD2* sequence.

**Molecular Weight Markers.** Molecular weight markers of *Hind*III-digested phage  $\lambda$  DNA were supplied by Boehringer Mannheim. Fragment sizes are 23.13, 9.42, 6.56, 4.36, 2.32, 2.03, 0.564, 0.125 kb. " $\lambda$  ladder" molecular weight markers were supplied by Boehringer Mannheim. Fragment sizes are 12.216, 11.198, 10.180, 9.162, 8.144, 7.126, 6.108, 5.090, 4.072, 3.054, 2.036, 1.636, 1.018, 0.517, 0.506, 0.396, 0.344, 0.298, 0.220, 0.201, 0.154, 0.134, 0.075 kb.

**Phage lysate** of  $\lambda$ Tn5(Km<sup>r</sup>) was supplied by Michael Stark, Dundee University. The phage is  $\lambda$ cl<sub>187</sub><sup>0am</sup> with an insertion of Tn5, possibly in the *RED* gene (this information has been lost during the complex history of the phage).

## CULTURE MEDIA

Yeast cells were grown with shaking at 30°C in conical flasks filled to 40% total volume in a complex **YEPD** medium comprising (per litre): yeast extract, 10 g; bacteriological peptone, 20 g; glucose, 20 g. **YEPG** medium has glycerol as the main carbon source, which yeast can only utilize by aerobic respiration. It will not support the growth of nuclear or mitochondrial *petites*. Medium comprising (per litre) glycerol, 30 g; bacteriological peptone, 20 g; yeast extract, 10 g. **YEPA** medium will only support growth by respiration. This medium was mainly used as a presporulation medium. Medium comprising (per litre) potassium acetate, 20 g; bacteriological peptone, 20 g; yeast extract, 10 g. **Minimal medium** (MM) consists of (per litre) glucose, 20 g; yeast nitrogen base (Difco, w/o amino acids and w/o ammonium sulphate), 1.7 g; ammonium sulphate, 5 g. **Leucine drop-out medium** (leucine-DO) consists of minimal medium with all amino acids except leucine, **Uracil drop-**







**out medium** (uracil-DO) consists of minimal medium with all amino acids except uracil, etc.

For growth on liquid media *Escherichia coli* was grown in **LB** (Luria broth) - bacto-tryptone 1%, yeast extract 0.5%, NaCl 0.5%. For growth on solid medium LB-Agar was used (LB media plus 1.5% bacto-agar). Cultures were incubated at 37°C.

## **RECOMBINANT TECHNIQUES**

### **Digestion by restriction enzymes**

DNA was digested by restriction enzymes using the following buffers from Boehringer Mannheim:

**A** Tris-acetate 33 mM, Mg-acetate 10 mM, potassium acetate 66 mM, dithiothreitol (DTT) 0.5 mM, pH 7.9.

**B** Tris-HCl 10 mM, MgCl<sub>2</sub> 5 mM, NaCl 100 mM, 2-mercaptoethanol 1 mM, pH 8.0.

**L** Tris-HCl 10 mM, MgCl<sub>2</sub> 10 mM, dithiothreitol 1 mM, pH 7.5.

**M** Tris-HCl 10 mM, MgCl<sub>2</sub> 10 mM, NaCl 50 mM, dithiothreitol 1 mM, pH 7.5.

**H** Tris-HCl 50 mM, MgCl<sub>2</sub> 10 mM, NaCl 100 mM, dithiothreitol 1 mM, pH 7.5.

The reaction mixtures were incubated 1-2 h at 37°C unless other requirements were needed. To inactivate restriction enzymes after a reaction was completed the reaction mixture was heated to 65°C for 15 min in the presence of 0.1% diethylpyrocarbonate (DEPC)







## **DNA Electrophoresis on Agarose Gel**

DNA samples were mixed with 1/10 volume of "gel loading buffer" (50% glycerol, 50 mM Tris-HCl pH 7.5, 100 mM EDTA, 0.02% bromophenol blue). Electrophoresis was performed in TAE buffer (40 mM Tris-acetate pH 7.5, 1 mM EDTA). Ethidium bromide was added to the gel at a concentration of 1  $\mu\text{g}/\text{ml}$ . After migration the gel could be photographed directly under UV light (302nm).

DNA fragments were purified from an agarose gel by cutting out the agarose containing the band and placing in a Biotrap (Schleicher and Schull). The fragments were immersed in TAE buffer for electrophoresis. The Biotrap works by trapping ethidium bromide and agarose by a first membrane, whereas the DNA was then trapped by a second membrane. After electrophoresis (the duration of which depends on the size of the fragment) the current was reversed for 10-15 sec to detach DNA adhering to the second membrane. The buffer between the two membranes was removed and an ethanol precipitation was performed to precipitate directly the DNA trapped in this chamber.

## **Alkaline phosphatase treatment of linear plasmid vector DNA**

Since during a cloning procedure, the vector was susceptible to religation to itself, it was possible to increase the percentage of insertions by treating the extremities of the vector with alkaline phosphatase. To do this, 5  $\mu\text{g}$  of linearised vector were resuspended in 50  $\mu\text{l}$  of the following mixture:

50 mM Tris-HCl pH 9, 1 mM  $\text{MgCl}_2$ , 0.1 mM  $\text{ZnCl}_2$ , 1 mM spermidine and 0.04 units of calf intestine alkaline phosphatase (Boehringer Mannheim). The mixture was incubated at 37°C for 30 min. At this point, another 0.04 units of alkaline phosphatase were added and incubation was continued for another 30 min. The quantity of enzyme necessary was calculated on the basis that 0.01 units were necessary to dephosphorylate 1 pmole of 5' DNA extremities,







which, in turn corresponds to 1.2  $\mu\text{g}$  of a 4 kb linearised vector. Inactivation of the alkaline phosphatase was carried out by incubation for 15 min at 65°C in the presence of 0.1% DEPC. The DNA was then treated with phenol, followed by phenol/chloroform, followed by chloroform and finally precipitated with ethanol. The DNA was then ready for ligation.

## **Ligation**

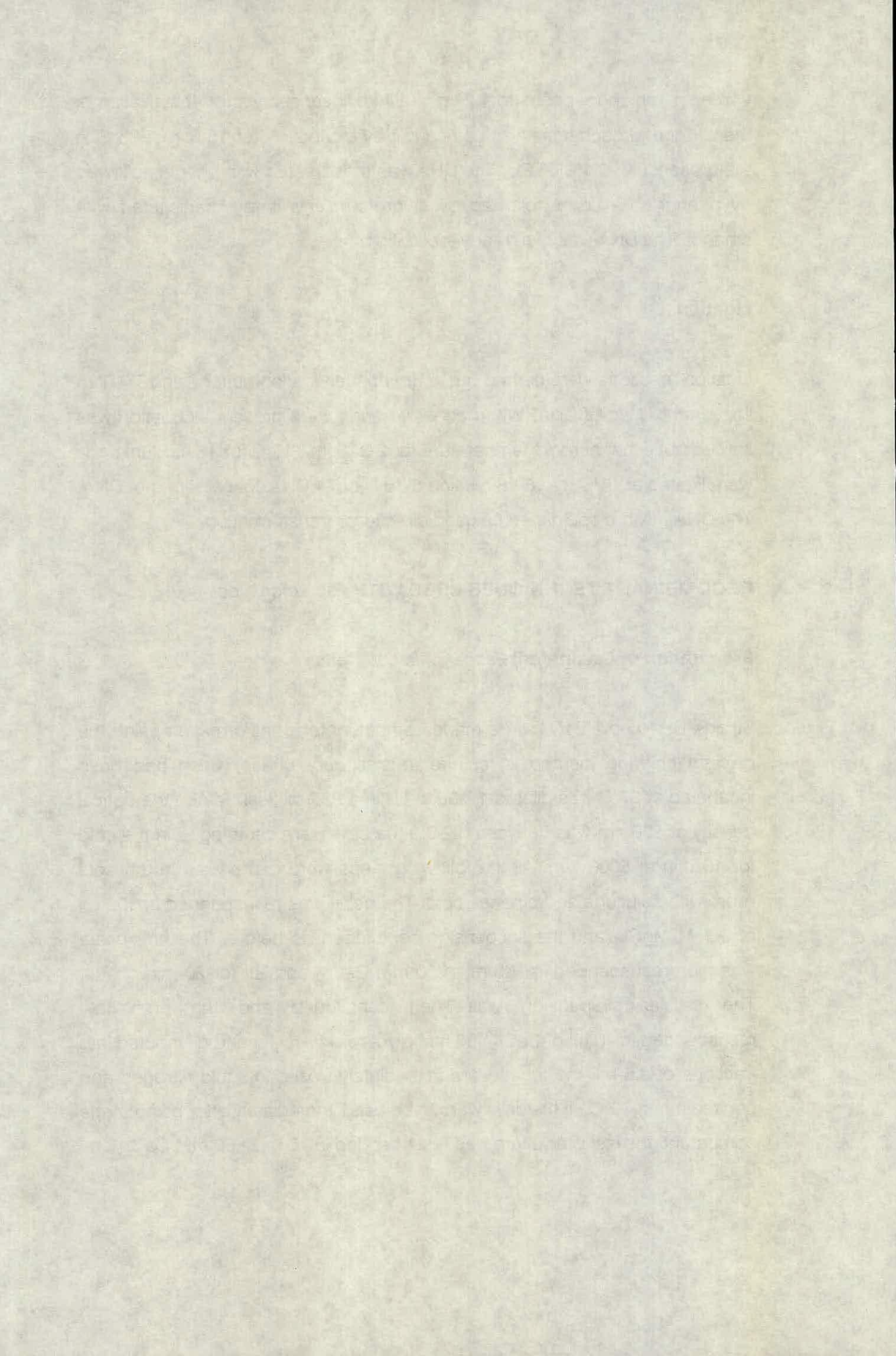
Ligation reactions were carried out in Boehringer ligation buffer using T4 DNA ligase at 1 U/ $\mu\text{g}$  of DNA. When cohesive ends were present incubation was carried out either at room temperature for 2-3 h or at 16°C for 16 h. Blunt ends were ligated at 16°C for 16-18 h using 5-10 U of T4 DNA ligase per  $\mu\text{g}$  of DNA. The DNA mixture could then be used directly for transformation.

## **RECOMBINANT TECHNIQUES USED WITH *Escherichia coli***

### **Preparation of Competent *Escherichia coli* Cells**

Strains DH1 and HB101 were made competent for transformation using the calcium chloride method. A culture in stationary phase which had been incubated at 37°C was diluted 1/200 in LB in a 250 ml flask. When the optical density at 550 nm was between 0.2-0.3 the cells were centrifuged in the cold for 10 min at 5000 X g. All the following steps were carried out in the cold room with solutions and pipettes cold. The pellet was resuspended in 100 ml of 0.1 M  $\text{MgCl}_2$  and the suspension centrifuged as before. The precipitate was then resuspended in 100 ml of 50 mM  $\text{CaCl}_2$  and left for 20 min on ice. The cellular suspension was then centrifuged and the precipitate resuspended in 10 ml of  $\text{CaCl}_2$  86 mM glycerol 14% (v/v) and distributed into fractions of 0.5 ml, the tubes were immediately frozen in liquid nitrogen and conserved at -70°C. If the cells were to be used immediately after being made competent, the last precipitate was resuspended in 10 ml of 50 mM  $\text{CaCl}_2$ .







## **Transformation of *Escherichia coli***

Cells were transformed as follows: 200  $\mu$ l of competent cells were mixed with DNA and left on ice for 15 min, after this the cells were exposed to heat shock at 42°C for 3 min. The cells were then returned to ice for 30 min after which 1 ml of LB was added and the tubes were left at 37°C for 1 h. After this period of expression, the cells were spread onto selective media. The efficacy of transformation for intact plasmid (0.1 - 2.0 ng) was from 3 to 5 x 10<sup>6</sup> transformants for DH1.

## **Identification of Recombinant Clones**

Since insertion of a DNA fragment into a vector happens at low frequency it is advantageous to be able to select for recombinant clones. Most vectors contain more than one gene for antibiotic resistance and the insertion of a fragment of DNA may inactivate this gene. It was therefore possible to replicate transformants onto a medium containing the corresponding antibiotic to detect sensitive clones.

Other plasmids (eg YEp352 series) not only contain the gene for ampicillin resistance but also contain the *lacZ* fragment from the *E. coli* lactose operon, into which a fragment containing many useful restriction enzyme sites has been inserted. Insertion of a fragment of DNA into the *lacZ* gene resulted in its inactivation. These plasmids were used with strains containing the deletion *lacZ* $\Delta$ M15 - e.g. CM5 $\alpha$ . When a bacterium contained the intact plasmid, there was complementation to form active  $\beta$ -galactosidase. Directly after transformation the bacteria were spread onto LB ampicillin, on which 50  $\mu$ l of X-Gal 2% in dimethylformamide and 50  $\mu$ l of IPTG had just been spread. IPTG is a free inducer of the *lac* operon and the X-Gal an artificial substrate of  $\beta$ -galactosidase which after hydrolyses generated a blue colour - recombinant clones stayed white.







## **Mini-preparations of Plasmidic DNA**

A method for quickly identifying a few dozen clones is the "boiling method" which is useful for a preliminary analysis of clones using restriction enzymes (Holmes and Quigley, 1981). From an overnight culture of 5 ml LB containing ampicillin (50  $\mu\text{g/ml}$ ) inoculated with a single colony, 1 ml was centrifuged in a bench centrifuge at about 13000 X g. The precipitate was resuspended in 250  $\mu\text{l}$  of STET buffer (50 mM Tris-HCl pH 8, 8% sucrose, 5% (v/v) Triton X100, 50 mM EDTA). The mixture was left on ice for 1 min in the presence of 20  $\mu\text{l}$  1mg/ml lysozyme. This was the lysis buffer. The tubes were then placed in a boiling water bath for 40 sec. The mixture was returned to ice for 15 min and then centrifuged for 15 min using a bench centrifuge. The viscous precipitate was removed from each tube using a sterile tooth pick. The DNA present in the supernatant was precipitated by adding 1 ml of ethanol and leaving for 15 min at  $-20^{\circ}\text{C}$ . After precipitation the DNA was resuspended in 50  $\mu\text{l}$  of water and 5-10  $\mu\text{l}$  were used for restriction enzyme digestion in the presence of RNase (40  $\mu\text{g/ml}$ ).

## **Large Scale Plasmid Preparation**

A transformant was grown on selective media overnight and 1 ml of this preculture was used to inoculate 500 ml of LB + ampicillin (50  $\mu\text{g/ml}$ ) which was then grown overnight at  $37^{\circ}\text{C}$  with shaking. The cells were then spun down at 6000 X g in a Sorval centrifuge. The precipitate was resuspended in 4.5 ml of TEG solution (25 mM Tris-HCl, 10m M EDTA, 50 mM glucose). The suspension was vortexed. 0.5 ml of lysozyme were added, the suspension was again vortexed briefly and then left on ice for 15 min. 15 ml of 0.2 NaOH/1% SDS were added. The tube was capped, mixed thoroughly by inversion and left on ice for 5 min. Next, 11.25 ml of 3 M NaAcetate pH 5 was added, the tube was inverted three or four times and then left on ice for a







further 30-60 min. Tubes were centrifuged for 40 min at 9000 X g in a Sorvall centrifuge. The supernatant was centrifuged through gauze and the DNA precipitated by addition of 45 ml of isopropanol - this was mixed well and left at room temperature for 5-10 min. The suspension was then centrifuged for 30 min at 9000 X g in a Sorvall centrifuge. The supernatant was discarded and the tube was drained upside down for 20 min. The pellet was resuspended in 3 ml of TE and transferred to a sterile Corex tube. The bottle was washed with a further 3 ml and combined. TE was then added to give a final volume of 8 ml. 7.75 mg of CsCl was added and left overnight at -4°C to precipitate any RNA. The tube was spun at 9000 X g for 30 min at 20°C. The supernatant was transferred equally to two Beckman centrifuge tubes and the tubes were balanced exactly with a solution of CsCl in TE at the same concentration as above. The tubes were centrifuged in a Beckman Ultracentrifuge for 17 h at 106,000 X g at 20°C.

The band of plasmid DNA (the lower band) was removed with a wide-bore syringe under UV light and the ethidium bromide was extracted using butan-1-ol. The DNA was then precipitated in the usual way.

## **RECOMBINANT TECHNIQUES USED WITH *Saccharomyces cerevisiae***

### **Preparation of competent yeast cells**

A preculture was grown overnight with shaking at 30°C of a yeast colony in YEPD. This culture was inoculated into fresh YEPD at an appropriate dilution to obtain after 15h of growth, about  $4 \times 10^6$  cells/ml (usually a dilution of about  $10^{-4}$ ). The cells were harvested by centrifugation for 5 min at 850 X g at room temperature in the rotor GSA 7000g. Cells were washed in 100 ml "buffer T" (10 mM Tris HCl pH 7.5, 10 mM  $\text{CaCl}_2$ , 1 M sorbitol) and centrifuged for 5 min at 850 X g and then cells were resuspended in 30 ml of "buffer T" (about  $7 \times 10^7$  cells/ml). 100  $\mu\text{l}$  of cells were removed and resuspended in 1 ml of water and the optical density was measured at 700







nm. 25 ml of a solution of zymolyase of concentration 5 mg/ml was added to the cells and incubation was carried out at 25-30°C. Each 15 min 100  $\mu$ l of cells were taken and diluted in 1 ml of sterile water; the optical density was measured. When the optical density reached 50% of the original value, (i.e. 50% sphaeroplasts) 100 ml of COLD "buffer T" was added to stop further zymolyase activity. Cells were centrifuged immediately for 5 min at room temperature. Sphaeroplasts were washed very gently three times with "buffer T" and each time were resuspended very carefully before centrifugation at 850 X g for 5 min. The cells were then resuspended in 50-100 ml of regeneration medium (YEPD/1 M sorbitol) and very gently agitated at 30°C for 1 h. Sphaeroplasts were centrifuged for 5 min at 850 X g and the precipitate was resuspended in 3 ml of "buffer T" (about  $10^9$  cells/ml). The competent yeast cells were then aliquoted into volumes of 0.3 ml in NUNC tubes and frozen at -70°C.

### **Yeast Transformation by the Sphaeroplasting Method**

Tubes containing 15 ml of MMS (sorbitol 1 M, casamino acids 0.5%, glucose 2%, YNB 1.7 g/l, ammonium sulphate 0.5%, agar noble 3% ) were melted in an autoclave, the required amino acid supplements were added and then the tubes were left in a water bath at 45°C. NUNC tubes containing 0.3 ml competent yeast cells were thawed on ice. 10  $\mu$ l of DNA in TE was placed in a sterile plastic tube (10 ml capacity) and 150  $\mu$ l of competent sphaeroplasts were added. This was mixed without vortexing and left at room temperature for 15 min. 1.5 ml of PEG solution (20% PEG 4000, 10 mM Tris-HCl pH 7.5, 10 mM  $\text{CaCl}_2$ ) was added, mixed without vortexing and then left at room temperature for 20 min. The suspension was then centrifuged for 10 min at 800 x g and the supernatant was carefully removed. The precipitate was resuspended in 750  $\mu$ l of solution T. A tube of MMS was removed from the water bath, wiped and the 750  $\mu$ l of cells were added to this tube, which was then vortexed very briefly. The contents of this tube were then spread onto







solid MMS medium containing required supplements.

### **Yeast Transformation by the Lithium Acetate Method**

A preculture was grown overnight to stationary phase at 30°C in YEPD. An inoculation of 1/100 in YEPD was made and this culture was grown at 30°C to an optical density of 0.4 at 700 nm (about  $5 \times 10^6$  cells/ml). 50 ml of cells were harvested by centrifuging at 850 X g for 5 min in a bench centrifuge. The pellet was resuspended in 5 ml TE and spun again. The pellet was again resuspended in 5 ml of TE and 208  $\mu$ l of 2.5 M Lithium acetate was added. The suspension was incubated for 1 h with gentle shaking at 30°C. 0.2 ml aliquots of cells were taken and mixed with DNA (1-10  $\mu$ g) in 0.1 ml. This was incubated for 30 min at 30°C. 0.7 ml 50% PEG-4000 was then added and the suspension was incubated for 1 h at 30°C after which it was heated to 42°C for 5 min. Cells were pelleted using a bench centrifuge, resuspended in 1 ml of sterile water and then spun down a second time. Finally the cells were resuspended in 0.1 ml of sterile water and plated onto selective medium.

### **Identification of Recombinant Clones**

Plasmids used to transform *Saccharomyces cerevisiae* contain selectable markers, usually genes involved in nucleotide or amino acid biosynthesis (e.g. *URA3*, *LEU2*) which may complement defective mutations (e.g. *ura3*, *leu2*) in the yeast strain being transformed.

### **Extraction of Yeast Chromosomal DNA**

A yeast strain was grown overnight to stationary phase in 10 ml of YEPD medium. The cells were washed once in water and resuspended in 0.5 ml buffer (0.9 M sorbitol, 0.05 M sodium phosphate buffer pH 7.5, 14 mM 2-mercaptoethanol) containing 2-5 mg/ml zymolyase and transferred to an







microcentrifuge tube. The tube was vortexed for 5 sec and then incubated at 37°C for 15-30 min, 50  $\mu$ l of 0.5 M EDTA pH 8.0, was added, the tube contents were vortexed briefly and 50  $\mu$ l 10% SDS and 100  $\mu$ l proteinase K solution (5 mg/ml) were added. The suspension was mixed well and incubated at 65°C for 15-30 min. The tube was allowed to cool, 500  $\mu$ l phenol/chloroform (1:1) was added and mixed well. The phases were separated with a 5 min spin in a bench centrifuge and the upper, aqueous, phase was transferred to a clean microcentrifuge tube. 500  $\mu$ l of ethanol was added and mixed by inversion. The precipitate was allowed to sediment then the ethanol was decanted off. The pellet was resuspended in 500  $\mu$ l TE (10 mM Tris-HCl, 1 mM EDTA pH 8.0), 5  $\mu$ l of RNase (2 mg/ml) was added and the suspension was incubated at 65°C for 15-30 min. The tube was allowed to cool after which 500  $\mu$ l of phenol/chloroform was added and was mixed well. The phases were separated by a 5 min spin in a bench centrifuge and the upper phase was transferred to a clean microcentrifuge. 500  $\mu$ l of ethanol was added and the contents of the tube were left overnight at 4°C. The supernatant was decanted off and 1 ml of ethanol was added. The tube contents were mixed by inversion and left at room temperature for 5 min. The tube was spun for 5 sec, the supernatant was removed and the DNA was dried in a vacuum desiccator. Finally, the pellet was resuspended in 50  $\mu$ l of TE.

### **Minipreparations of Yeast Plasmid DNA**

The strains containing the plasmid to be extracted, were grown in 2 ml of selective medium: 1-2 days of growth were necessary after inoculation. The culture was transferred to a microcentrifuge tube and centrifuged with a bench centrifuge for 5 min. The supernatant was removed and the cells resuspended in the residual liquid (about 150  $\mu$ l). 200  $\mu$ l of "Triton Solution" was added (100 ml of solution contains 2 ml of Triton X-100, 10 ml of 10% SDS, 10 ml of 1 M NaCl, 10 ml 1 M Tris pH 8, 200 ml 0.5 M EDTA pH 8), 200  $\mu$ l of phenol/chloroform and 0.3 g of glass beads. This was vortexed for two







min minimum and then centrifuged for 5 min. The supernatant (about 500  $\mu$ l) was then precipitated with 50  $\mu$ l of 5 M potassium acetate and 1 ml of cold ethanol left at room temperature for 2 min. This was then centrifuged and the precipitate was resuspended in 20  $\mu$ l. 10  $\mu$ l volumes were used to transform *E. coli*.

## METHOD FOR Tn5 TRANSPOSON MUTAGENESIS

The phage used was lambda cl<sub>857</sub>O<sup>am</sup> with an insertion of Tn5, possibly in the *red* gene (this information was lost during the complex history of the phage at Leicester University). Phage was supplied by Dr Michael Stark of Dundee University. Because of the *amber* mutation in the O gene, the phage must be propagated in a *supE* strain (e.g. C600, 5K).

### Preparation of a High Titre Phage Lysate

An overnight 5 ml culture of *E. coli* strain 5K was grown in LB containing maltose (0.2%). 10 mM MgCl<sub>2</sub> was added to the overnight culture and infections were set up by adding 100  $\mu$ l phage (10<sup>6</sup> pfu/ml: phage stock diluted in phage buffer) to 50  $\mu$ l cells. Incubation at room temperature for 10 min allows the phage to absorb. 6 ml of BBL top agar (1% Trypticase, 0.5% NaCl, 1% agar) cooled to 45-50°C was mixed with the cells and phage and poured onto solid BBL plates. This was allowed to set and was then incubated at 37°C. After 3-4 h the plates were inspected for the onset of lysis i.e. the appearance of plaques. When the plate had taken on a "lacy" appearance the top layer of agar was scraped off using a 1 ml pipette and put into a glass universal. The agar was spun for 15 min at 10,000 X g and the aqueous supernatant was removed to another universal and stored over chloroform.









## Mutagenesis using lambda:Tn5

The procedure depends upon infecting a *sup<sup>O</sup>* target strain and then plating out for kanamycin resistant (*Km<sup>r</sup>*) colonies. Under the conditions used the phage can neither lysogenise (*cl<sub>857</sub>* at 37°C) nor replicate (*O<sub>am</sub>* in a *sup<sup>O</sup>* background) so that *Km<sup>r</sup>* colonies reflect transposition events.

The plasmid to be mutagenised was first transformed into the *sup<sup>O</sup>* strain, 549. The transformed strain was grown overnight in LB plus 50 µg/ml each of ampicillin and kanamycin. The bacteria were diluted 1/100 in LB and antibiotics plus 0.2% maltose and grown to an optical density of 0.5 at 550 nm. 5 ml of the cells were harvested and resuspended in 2.5 ml of phage buffer, then 1 ml of cells were added to 1 ml of phage and incubated at room temperature for 10 min. 4 ml of LB were added and the mixture was incubated at 42°C for 10 min, followed by incubation at 37°C for 30 min. Cells were spun down, resuspended in 100 µl of supernatant and plated onto solid LB plus ampicillin and kanamycin. Plates were incubated at 37°C overnight. Colonies were washed off using LB and used to inoculate LB plus kanamycin and ampicillin; the cells were grown overnight. Plasmid DNA was prepared by the "boiling miniprep" procedure and used to transform the strain 5K to ampicillin and kanamycin resistance. Single transformants were screened for the location of the Tn5 insertion.

## HYBRIDIZATION ON NITROCELLULOSE

### Hybridization to Chromosomes Separated by Pulse-field Electrophoresis

A yeast chromosome transfer of yeast chromosomes separated by the CHEF gel system on nitrocellulose was donated by Dr. D. H. Williamson from The National Institute for Medical research, Mill Hill, London.







## Radioactive Labelling of DNA

DNA was labelled using the Amersham Nick Translation Kit. 100 ng of DNA were treated with a volume of 20  $\mu$ l of buffer containing 50 mM Tris-HCl pH 7.8, 5 mM MgCl<sub>2</sub>, 10 mM  $\beta$ -mercaptoethanol, dGTP, dATP, dTTP - 80  $\mu$ l of each, [ $\alpha$ P<sup>32</sup>]-dCTP 0.925 MBq (30 TBq/mmol), 0.5 U *Escherichia coli* DNA polymerase I, 10 ng of DNase I. The incubation reaction was carried out for 2 h at 16°C, the reaction was stopped with 2  $\mu$ l of 0.5 M EDTA. The DNA was purified by passing down a column adding 3 X 400  $\mu$ l of TE buffer and collecting aliquots in three Ependorff tubes. The contents of the second tube contained the radiolabelled DNA and this aliquot was used to hybridize to the nitrocellulose membrane. Specific radioactivity obtained was in the order of  $5 \times 10^8$  dpm/ $\mu$ g of DNA. Before use, the probe was denatured by heating to 100°C for 5 min followed by rapid cooling in ice.

## Hybridization with a radioactive probe.

**Prehybridization:** the nitrocellulose membrane was treated enclosed in a plastic sac with a large excess of hybridization buffer (5 X SSC; 50 mM Na/Na<sub>2</sub>PO<sub>4</sub> pH 7; SDS 0.1%; EDTA 5 mM; 100  $\mu$ g/ml denatured salmon sperm DNA; 2 X Denhart's solution) for several hours at 42°C.

**Hybridization:** The buffer which served for the prehybridization was replaced by fresh buffer and the radioactive probe was added after dilution in 1 ml of hybridization buffer. The total volume was kept to a minimum and air bubbles were eliminated. The hybridization was carried out at 42°C for 16-40 h. The membranes were then rinsed two times for 5 min at room temperature in a rinsing solution (0.2 X SSC; SDS 0.2%), three times for 30 min at 60°C in the same rinsing solution and 2 times for 5 min at room temperature in 2 X SSC. The membranes were dried and then autoradiographed at -70°C in cassettes with screen intensifiers - Kodak X Omatic - on the films Kodak X ARS.







## **SEQUENCING TECHNIQUES**

DNA sequence was determined by the Sanger technique (Sanger *et al.*, 1977) using vectors derived from phage M13 (Messing, 1983) and also by using double stranded sequencing with PKS/PSK vectors.

### **Single Stranded Sequencing Cloning in vector M13**

Vectors: the vectors used were M13 mp18 and mp19 (Yanisch-Perron *et al.*, 1985).

The DNA of phage M13 can be obtained either in the single stranded form (phage form) or in the double stranded form (the replicative form present in infected bacteria). For cloning, the double stranded form was used. Ligations were carried out as for normal plasmids.

### **Transfection of *E. coli*.**

All the steps of transfection were identical to an ordinary transformation until heat shock. After heat shock, the transformed cells were mixed with a solution containing 200  $\mu$ l of CM5 $\alpha$  in exponential phase of growth, 40  $\mu$ l of 10 mM IPTG, 40  $\mu$ l of 2% X-Gal in dimethylformamide. This solution was then mixed with 3 ml of H top-agar (1% bacto tryptone, 0.8% NaCl, 0.8% agar) maintained as liquid at 42°C. After mixing, the contents were rapidly spread onto plates and incubated at 37°C overnight. White plaques were selected since they contained M13 with an insert disrupting the *lacZ* gene.

### **Preparation of Single Stranded DNA of phage M13**

About 18-24 h after transformation, each plaque containing recombinant phages (white plaques) was sampled by picking with a tooth pick and was used to inoculate 1.5 ml of 2TY medium previously inoculated 1/100 with a







saturated culture of CM5 $\alpha$ . The tubes were shaken at 37°C for 5h, after which the culture was centrifuged 3 min in a bench centrifuge (130000 X g), the supernatant, containing the phages was removed and centrifuged a second time to eliminate as much as possible of the bacteria present. 200  $\mu$ l of 20% polyethylene glycol "600", 2.5 M NaCl were added, and after mixing this, the solution was left 15 min at room temperature. The phages were sedimented by centrifugation (5 min) the supernatant was removed and after a second centrifugation of 2 min the tube was wiped to eliminate all traces of polyethylene glycol. The viral sediment was then resuspended in 100  $\mu$ l of TE (10 mM Tris-HCl pH 7.5, 1 mM EDTA) and extracted with 50  $\mu$ l of phenol saturated in TE. After centrifugation, the aqueous phase was removed, 10  $\mu$ l of 3 M NaAcetate were added and 250  $\mu$ l of absolute alcohol. After 1 h or more at -20°C the precipitated DNA was centrifuged for 15 min at 130000 X g, rinsed in ethanol and dried; the precipitate was then redissolved in 30  $\mu$ l of TE.

### **Analysis of M13 clones**

Before using the DNA for the sequence reactions, gel electrophoresis was carried out in the presence of a known quantity of M13 phage DNA without insert. This permitted detection of the presence of an insert by showing shorter migration than the control DNA, and at the same time permitted an estimate of the concentration of the single stranded DNA. It was also possible to identify clones having inserts in the reverse orientation by hybridization. For this, 2-3  $\mu$ l of single stranded DNA of each of the clones were incubated 15 min at 65°C in 10  $\mu$ l of TE solution, 250 mM NaCl, and left to cool to room temperature. The mixture was then separated on an agarose gel; from clones having the same sequence but in opposite orientation the single stranded DNAs hybridized to form a structure in the form of an "8" which migrated more slowly than the circular single stranded DNA.







## Reaction Sequences

Sequence was determined by the Sanger method using the Amersham sequencing kit. The oligodeoxynucleotide used as primer was the "M13 sequencing primer 17-mer" (5'GTAAACGACGGCCAGT-3') and the labelling was carried out with [ $\alpha^{35}\text{S}$ ]-ATP (22 TBq/mmol). The working solutions containing the mixture dCTP + dGTP + dTTP + ddXTP were supplied by Pharmacia.

The primer (1.5 ng) was hybridized with about 200 ng of single stranded DNA in 10  $\mu\text{l}$  of reaction buffer (Tris-HCl 10 mM pH 8.5;  $\text{MgCl}_2$  10 mM) by heating the mixture to 60°C for 20 min and by letting it cool gently to room temperature. 1.5  $\mu\text{l}$  of [ $\alpha^{35}\text{S}$ ]-ATP and 1  $\mu\text{l}$  of Klenow fragment (1U). The mixture was divided into four tubes marked T,C,G and A (each 2.5  $\mu\text{l}$ ). In each of these tubes, 2  $\mu\text{l}$  of the working solution were added. This was incubated for 20 min at 25°C then 2  $\mu\text{l}$  of the "chase" solution was added (0.5 mM of each of the 4 deoxynucleotides) and the polymerisation was followed for a further 15 min. the reaction was stopped by 2  $\mu\text{l}$  of loading buffer (96% deionised formamide, 20 mM EDTA, 0.03% bromophenol blue, 0.03% xylene cyanol). The reaction mixtures were stored at -20°C until they were used for gel electrophoresis.

## Sequencing Gels

The acrylamide solution was prepared in the following way:

38 g of acrylamide and 2 g of bisacrylamide were dissolved in 100 ml of distilled water, the solution was deionised by agitation in the presence of ion exchange resin (Mixed Bed Resin AG 501-X8, Bio-Rad) and filtered. Two types of buffers were used to prepare the gels:

TBE buffer pH 8.3 (Tris-Borate pH 8.3 89 mM. 2.5 mM EDTA) and buffer TBE pH 8.8 (Tris-Borate pH 8.8 130 mM). The gels were prepared by mixing in a final volume of 50 ml: 7.5 ml of 40% acrylamide (final concentration 6%), 5 ml







of 10 X TBE buffer, 20 ml of water and 21 g of urea (final concentration 7 M). The mixture was gently warmed to aid dissolution of the urea, 300  $\mu$ l of 10% ammonium persulphate and 50  $\mu$ l of TEMED were added and the gel was immediately left to set. Gels used were 20 x 40 cm with a thickness of 0.4 mm. Each sequencing reaction was loaded onto each of the two gels, one gel was for a short migration (stops when the bromophenol blue reaches the bottom of the gel) in buffer TBE pH 8.3, and a long migration in TBE pH 8.8 buffer, where the xylene cyanol was at the bottom of the gel, 2  $\mu$ l of loading buffer were deposited and the migration was followed until the bromophenol blue was at the bottom of the gel. The electrophoresis was carried out at a constant rate of 18 to 23 mA (1100-1300 Volts), whether the migration was short (about 2 h) or long (about 6 h). With the two gels it was possible to read about 300-350 nucleotides per sequence reaction. After migration the gel was fixed for about 15 min in a solution of 10% acetic acid, 10% methanol. Next, the gel was dried under vacuum at 80°C (Hoeffer gel drier) and autoradiographed for 24-48 h on Kodax X AR5 film.

### **Treatment of information**

The Max Plank data base (MIPS) program was used for translations of nucleic acid sequences and for identifying optimum alignment between similar proteins. The alignment algorithm used was based on the work of Dumas and Ninio, (1982), Needleman and Wunsch, (1970), and Wilbur and Lipman, (1983). Higher alignment scores between two sequences indicate greater similarity.

### **Double stranded sequencing**

Double stranded recombinant DNA was isolated from white plaques using the same protocol for isolating single-stranded M13 (see above). Care was taken to eliminate all proteins by carrying out several phenol/chloroform







extractions. Using the Pharmacia protocol, an optimal ratio of matrix/primer was needed. The concentration of the primer solution was adjusted until 2  $\mu$ l contains the mass of primer needed to obtain the ideal primer/matrix chosen. The concentration of the matrix was adjusted to 1.5-2  $\mu$ g in 8  $\mu$ l. To denature the double stranded matrix 2  $\mu$ l of 2 M NaOH was added and the mix was centrifuged for 10 min after being vortexed gently. Then, 3  $\mu$ l of 3 M NaAcetate pH 4.5 and 7  $\mu$ l of distilled water were added. 60  $\mu$ l of 100% alcohol were added and mixed and left at -20°C for 15 min. This was then centrifuged for 10 min. The precipitate was washed in 70% cold ethanol and recentrifuged, then dried and redissolved in 10  $\mu$ l of distilled water. 2  $\mu$ l of hybridization buffer and 2  $\mu$ l of primer solution were added and this was incubated at 37°C for 20 min to hybridize the primer. The tube was left at room temperature for 10 min. If the sequencing reactions were not carried out on the same day, then the hybrid of primer/matrix can be stored at -20°C. The reaction sequencing reactions were carried out as described for single stranded DNA above.

## **RESPIRATION MEASUREMENTS**

An oxygen electrode sensor was used to measure the maximum rates of respiration of yeast cells in the stationary phase of growth. 200 ml of cells were grown to stationary phase at 30°C in YEPD medium. A sample of cells was transferred to a cuvette in the oxygen electrode sensor and glucose or ethanol added. An oxygraph was produced which measures the rate of oxygen uptake and thus reflects the rate of respiration. Measurements are given in nmoles O<sub>2</sub>/min/mg dry weight.







## RESULTS







## MOLECULAR CHARACTERIZATION OF THE *SSPD1* SEQUENCE







## INTRODUCTION

Dawes noted that yeast strains containing any one of the *spd* mutant alleles show the highest levels of sporulation on non-fermentable substrates, yet, the *SPD* genes were found to be non-allelic to any of the known mutations giving rise to similar effects, e.g. *cdc25*, *ras2*, *cyr1*, (Janice Doull, personal communication). Dawes began a project to isolate the *SPD* genes with the aim of studying their structure and function in greater detail.

Janice Doull used an *spd1* diploid strain 407.10D (*spd1/spd1*, *leu2/leu2*) to screen a YEp13 based gene bank (donated by Kim Nasmyth) for the *SPD1* gene. The selection used depended on the fact that *spd* mutants do not grow well on complete media containing glycerol as the sole carbon source but stop in the G1 phase of growth and immediately begin to sporulate. The selection used to screen the bank for this gene consisted of transforming the *spd1* mutant from leucine auxotrophy to leucine prototrophy using the method of sphaeroplast transformation and then individual transformants were picked, using sterile toothpicks, to YNB plates containing all necessary amino acids except leucine (leucine-DO). About 50 individual colonies could be picked onto one plate. Each plate was then replica plated to a YEPG plate (complete media with glycerol as the carbon source), then growth was compared with that of the *spd1* untransformed strain and that of a wild-type strain. Using this technique, more than fifteen thousand transformants were screened using the Nasmyth gene bank with an average insert size of about 5 kb.

Having picked 9 colonies which showed better than background growth on YEPG, segregation was tested for glycerol growth and plasmid loss. This was achieved by streaking a transformant onto non-selective medium YEPD (complete media with glucose as the carbon source), allowing colonies 2-3 days to grow, and then picking about 100 of these individual colonies onto YEPD plates (about 50 colonies per plate). These plates were then simultaneously replica plated onto leucine drop-out plates and YEPG plates







and ability to grow on glycerol was compared with plasmid retention/loss. In one of these transformants the ability to grow on glycerol was concomitantly lost with plasmid loss for 100 of the colonies tested. Plasmid DNA was isolated from the yeast and used to transform *E. coli*. Several transformants were obtained. Mini plasmid preps were carried out and individually used to transform yeast *spd1* mutants. In the cases tested, the plasmid DNA successfully restored growth on glycerol. The DNA was amplified in the *E. coli* strain HB101 and the plasmid partially characterized by restriction enzyme mapping. This plasmid was named pJLD1 and contained an insert of about 4.8 kb which will be referred to as *SSPD1* (suppressor of *spd1*). The original restriction map of the *SSPD1* sequence is shown in Figure 6.

## RESULTS

### DOES pJLD1 COMPLEMENT THE HYPERSPORULATION PHENOTYPE OF *spd* MUTANTS?

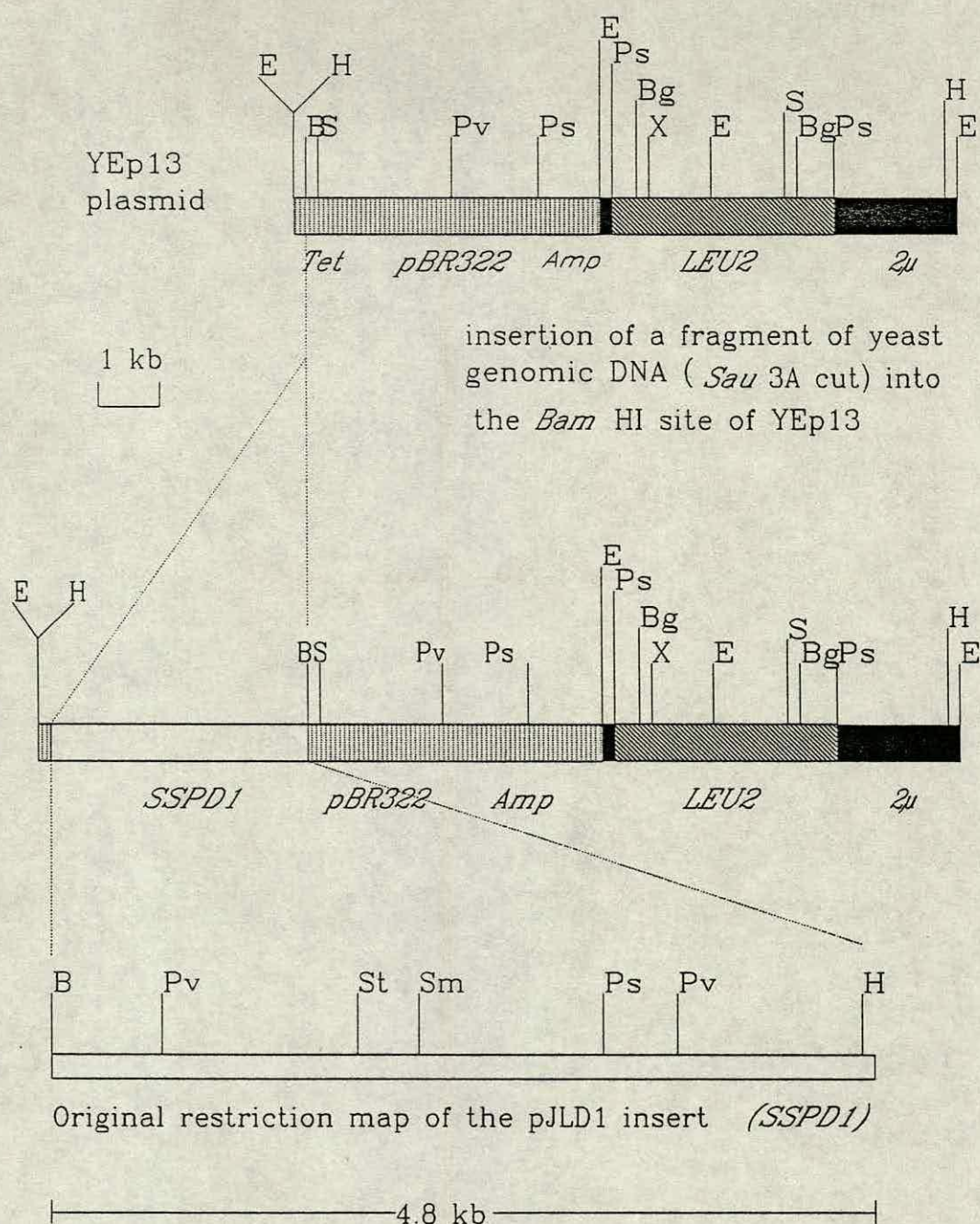
The plasmid pJLD1 does not fully restore the growth of an *spd1* mutant on YEPG. There are two possible reasons for this. Firstly, an isogenic wild-type strain was not available and so differences in growth on YEPG could not be readily attributed to the difference of a single gene. Secondly, the medium YEPG does not confer a selective pressure for the pJLD1 plasmid and consequently, transformants may readily lose their plasmid on cell division. Examination of cells by light microscopy showed that a smaller proportion of cells were sporulating in transformed colonies than in untransformed *spd1* colonies but more cells were sporulating in a transformed colony compared with a wild-type control, S41 (Table 3).

To test directly whether the pJLD1 plasmid was itself responsible for inhibiting sporulation, 20 individual tetrads and 20 budding cells from a pJLD1(410:2C)







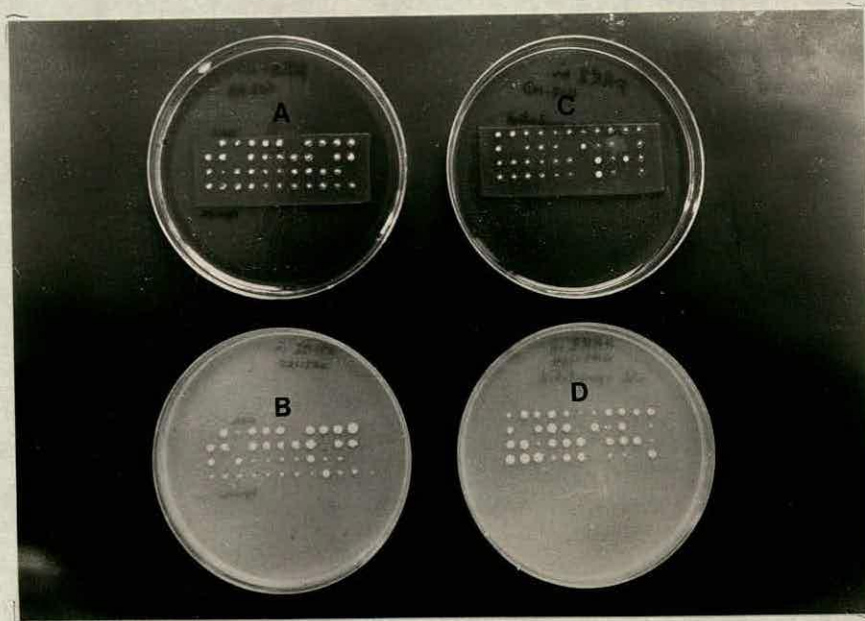


**Figure 6:** Origin and restriction map of the pJLD1 insert (*SSPD1*). Abbreviations used are as follows: (Bg) *Bgl*II; (B) *Bam*HI; (E) *Eco*RI; (H) *Hind*III; (Ps) *Pst*I; (Pv) *Pvu*II; (S) *Sal*I; (Sm) *Sma*I; (St) *Stu*I; (X) *Xho*I.









**Figure 7:** Individual budding cells and tetrads tested for the presence of plasmid.

In each of the plates shown above, the top two rows of colonies have been grown from vegetatively dividing cells obtained by dissection, while the bottom two rows have been grown from sporulating cells. Plate B was replica-plated from A, while plate D was replica-plated from C.

<b>MEDIUM</b>	<b>PLATE</b>
YEPD	A, C
leucine-DO	B, D
<b>STRAIN</b>	<b>PLATE</b>
410:2C(pJLD1)	A, B
410:2C(YEp13)	C, D

From plate B it appears that sporulation arises mainly due to cells having lost the plasmid pJLD1. Plate D shows that sporulation occurs whether the plasmid YEp13 is present or not.







**Table 3:** Percentage sporulation of different *S. cerevisiae* strains on YEPG solid medium after incubation at 30°C for 48 h. 200 cells were counted for each strain.

STRAIN	% SPORULATION AFTER 48 h ON YEPG SOLID MEDIUM
S41	10
59.4A	75
410:2C	71
410:2C(YEp13)	60
410:2C(pJLD1)	25

**Table 4:** Plasmid pJLD1 may inhibit sporulation in an *spd1* mutant.

	STRAINS	
	410:2C(pJLD1)	410:2C(YEp13)
% OF COLONIES DERIVED FROM INDIVIDUAL TETRADS HAVING LOST THEIR PLASMID	80	25
% OF COLONIES DERIVED FROM INDIVIDUAL VEGETATIVELY GROWING CELLS HAVING LOST THEIR PLASMID	5	25







colony were individually picked onto YEPD with the aid of a microscope and dissection needle (Fig. 7). Colonies grown from these individual tetrads and budding cells were tested for their ability to grow on leucine drop-out medium (leucine-DO). It was found that of the 20 colonies derived from individual tetrads, 16 could not grow on leucine drop-out medium suggesting that in 80% of sporulating cells there was no pJLD1 plasmid present. Of 20 colonies derived from individual budding cells only 1 did not grow on leucine drop-out media suggesting that in only a few budding cells there was no plasmid present. 410:2C(YEp13) was used as a control; this time, of 20 colonies derived from tetrads 5 (25%) were found not to grow on leucine drop-out medium, and for colonies derived from budding cells again 5 (25%) failed to grow on leucine drop-out media (Figure 7, Table 4). The conclusion was therefore drawn that the pJLD1 sequence may play a role in inhibiting sporulation when present in an *spd1* mutant.

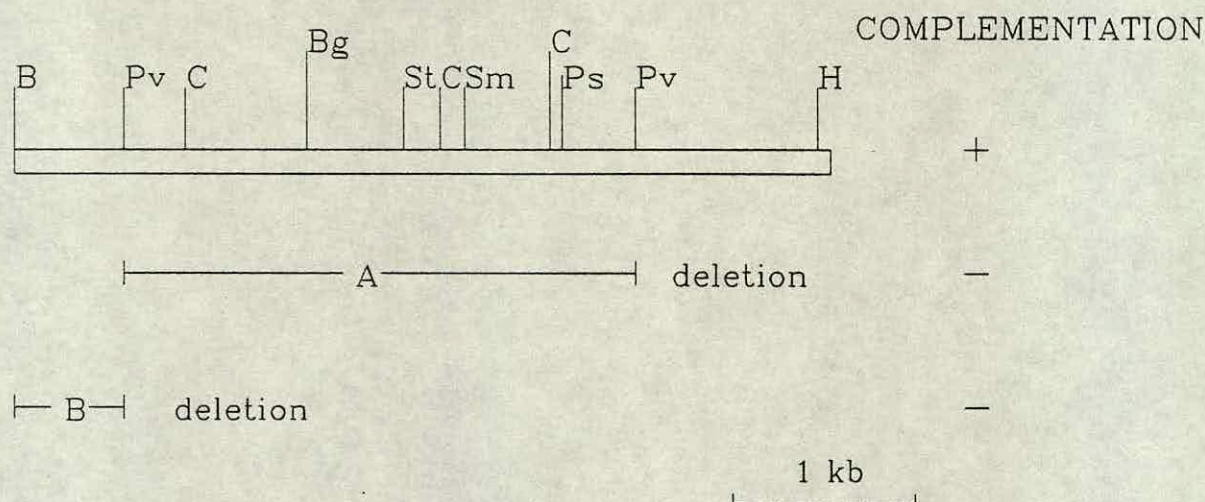
#### **DETERMINING THE COMPLEMENTING REGION OF THE *SSPD1* SEQUENCE USING DELETIONS.**

A more complete restriction map for the *SSPD1* sequence is shown in Figure 8. Two deletion plasmids were constructed by carrying out partial *PvuII* digestions of pJLD1 followed by religation. The first, named pAR2, contained a deletion (A) of 3.0 kb, while the second, named pAR3 contained a smaller deletion (B) of 0.9 kb (Fig. 8). The two resulting plasmids were both used to transform the *spd1* strain 410.2C but failed to complement the growth and sporulation defect of the *spd1* mutant. Dissection was carried out as above to determine whether the ability to stop sporulation was still present, but in both cases equal numbers of sporulating and budding cells lacked plasmid suggesting that the deletion plasmid did not inhibit sporulation. From these results it was concluded that the complementing region of pJLD1 overlaps the *PvuII* site located 0.9 kb from the *Bam*HI site. (Fig. 8)









**Figure 8:** A more complete restriction map of the *SSPD1* sequence. Abbreviations used are as follows: (Bg) *Bgl*II; (B) *Bam*HI; (C) *Cla*I; (H) *Hind*III; (Ps) *Pst*I; (Pv) *Pvu*II; (Sm) *Sma*I; (St) *Stu*I. Enzymes which were found not to cut are *Sal*I, *Xho*I, *Eco*RI, *Xba*I, *Sst*II, *Kpn*I, *Pvu*I.

Two different *Pvu*II deletion plasmids were made - pAR2 was derived from pJLD1 by deleting the 3.0 kb sequence (A), while pAR3 was derived from pJLD1 by deleting a 2.8 kb sequence, 0.9 kb of which was present in the *SSPD1* sequence (B). Neither of these two deletion plasmids were capable of suppressing the sporulation and growth defects of an *spd1* mutant on YEPG medium. It was therefor assumed that the suppressing region of the *SSPD1* sequence overlaps the first *Pvu*II site.







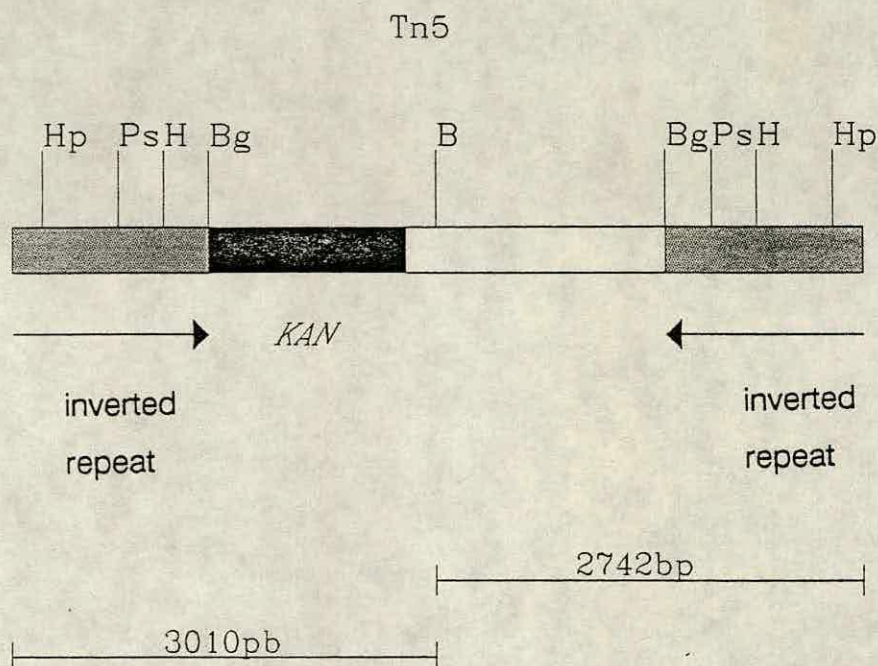
## Tn5 MUTAGENESIS USED TO LOCALIZE THE COMPLEMENTING REGION OF THE *SSPD1* SEQUENCE.

The technique of Tn5 mutagenesis was used to randomly disrupt *SSPD1* and localize more precisely the complementing region of the sequence. Lambda phage carrying Tn5 was provided by Michael Stark and consisted of lambda cl<sub>857</sub> O<sup>am</sup> with an insertion of Tn5 thought to be in the *red* gene. A map of the Tn5 plasmid is given in Figure 9. The *E. coli sup<sup>O</sup>* strain NM554 was transformed to ampicillin resistance with pJLD1, grown overnight and infected with lambda Tn5 phage lysate. Transposition events were selected using kanamycin resistance. Over 1000 colonies were pooled and a plasmid preparation carried out. The pJLD1-Tn5 transposition bank was used to transform the *E. coli* strain 5K and mini plasmid preparations were carried out on individual colonies. Restriction analysis was carried out using *Bam*HI/*Hind*III double digests to identify transpositions into the *SSPD1* sequence - this was based on the assumption that if Tn5 has integrated into the *SSPD1* sequence, then the 4.8 kb band should disappear in most instances whereas the 10.7 kb band of YEp13 should still be present. Plasmids shown to have Tn5 integrated into the *SSPD1* sequence were then digested with *Bam*HI to estimate the position of the integrative event relative to the *Bam*HI site marking the *SSPD1*/YEp13 junction. From analysis of about 100 Kn<sup>R</sup> Amp<sup>R</sup> colonies the *spd1* complementing region of pJLD1 was shown to be a region of about 1.74 - 2.62 kb as indicated in Figure 10. Since the *Bam*HI site within the Tn5 transposon is not exactly in the centre (Fig. 9) and measurements were not made to determine its orientation within the *SSPD1* sequence these results are only indicative of the size and position of the complementing area of the *SSPD1* sequence and do not represent the exact limits of the suppressor gene.

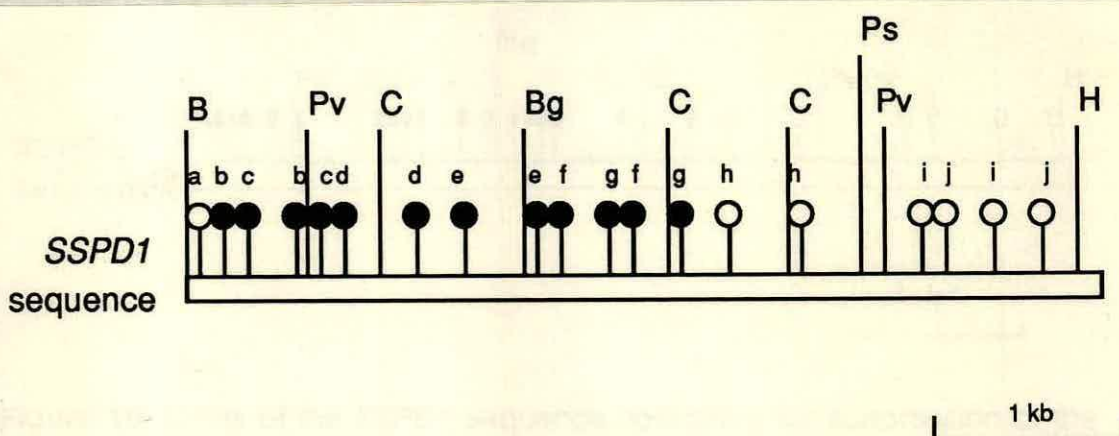








**Figure 9:** Map of the transposon Tn5 which contains a the gene coding for kanamycin resistance (*KAN*). Abbreviations used are as follows: (Bg) *Bgl*II; (B) *Bam*HI; (H) *Hind*III; (Hp) *Hpa*I; (Ps) *Pst*I.



**Figure 10:** Limits of the *SSPD1* sequence necessary for suppression of the *spd1* mutant yeast strain 410:2C for sporulation and growth on YEPG, as estimated by Tn5 disruption data. Black "lollipops" represent positions of Tn5 integration which result in loss of complementing activity of the *SSPD1* sequence. White "lollipops" represent positions of Tn5 integration which do not destroy complementing activity of the *SSPD1* sequence. Each single Tn5 insert is depicted as a pair of "lollipops" (300 bp apart) designated by the same letter to indicate the two possible orientations of the Tn5 transposon insertion. Abbreviations used are as follows: (Bg) *Bgl*II; (B) *Bam*HI; (C) *Cl*aI; (H) *Hind*III; (Ps) *Pst*I; (Pv) *Pvu*II.



РvIII.

used are as follows: (Bd) BqIII; (B) BqWNI; (C) CqI; (H) HqIII; (Ps) PqII; (Pv) not destroy complementing activity of the 22pD1 sequence. Approximate sequence. White "pillbobs" represent positions of the integration which do not integration which result in loss of complementing activity of the 22pD1 estimated by the disruption data. Black "pillbobs" represent positions of the 22pD1 mutant yeast strain 410:5C for sporulation and growth on YEPG, as Figure 10: limits of the 22pD1 sequence necessary for suppression of the





## THE *SSPD1* SEQUENCE IN THE YCp50 PLASMID VECTOR

The *SSPD1* sequence was transferred, as a *Bam*HI/*Hind*III fragment, from the high copy plasmid vector YEp13 to the low copy vector YCp50 (Fig. 11). This new vector, pARC1, was used to transform *spd1* diploids and transformants were tested for sporulation and growth on YEPG solid medium. It was found that pARC1 did not complement *spd1* mutants for either the growth on glycerol or hypersporulation phenotypes.

## DOES pJLD1 COMPLEMENT THE HYPERSPORULATION PHENOTYPE OF *spd3* OR *spd4* MUTANTS?

The pJLD1 plasmid was used to transform *spd3* and *spd4* mutant yeast strains, named 391 and 417 respectively. No visible suppression of sporulation or growth defects of these strains by pJLD1 was observed.

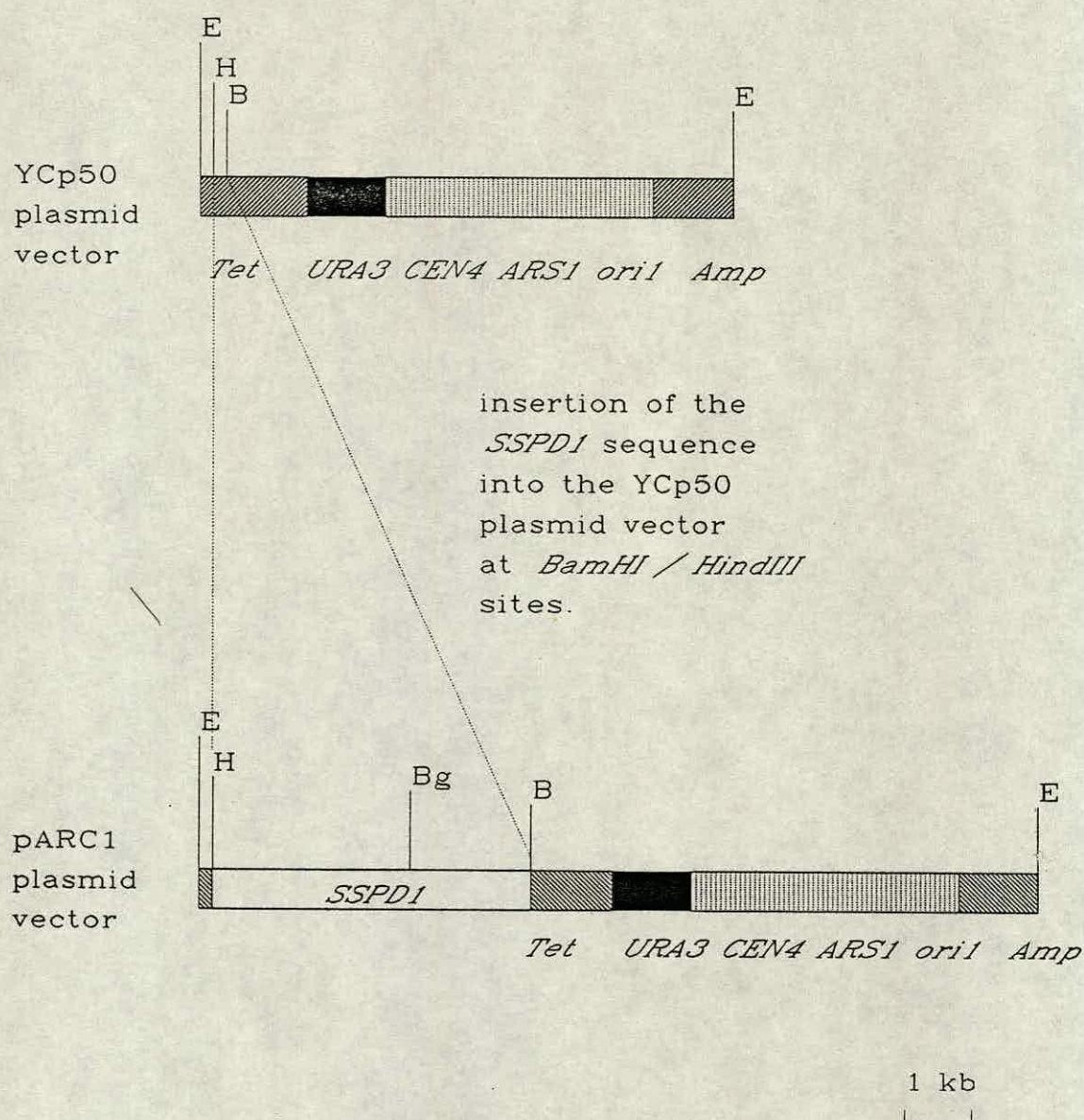
## CHROMOSOMAL LOCALIZATION OF THE *SSPD1* SEQUENCE

A nitrocellulose filter containing separated yeast chromosomes from strain YP148 (provided by Phil Heiter, who had engineered a split at the *RAD2* locus in chromosome VII to resolve it away from chromosome XV, Vollrath *et al.*, 1988). The genotype of the strain YP148, before engineering, was *MATa ura3-52 lys2-801 ade- his7 trp1-d1*. After engineering the phenotype was changed to *Trp*<sup>+</sup> and *Ura*<sup>+</sup>. Plasmid pEI6 was nick translated and used as a probe (Fig. 12) and three bands were found on the autoradiograph (Fig. 13). Two of these bands corresponded to hybridization of pBR322 sequences, present as a result of engineering the split in chromosome VII and also present on the pEI6 plasmid. The third band corresponded to hybridization of the *SSPD1* sequence with its chromosomal homologue and this band was shown to correspond to the position of chromosome VIII (Fig. 13).







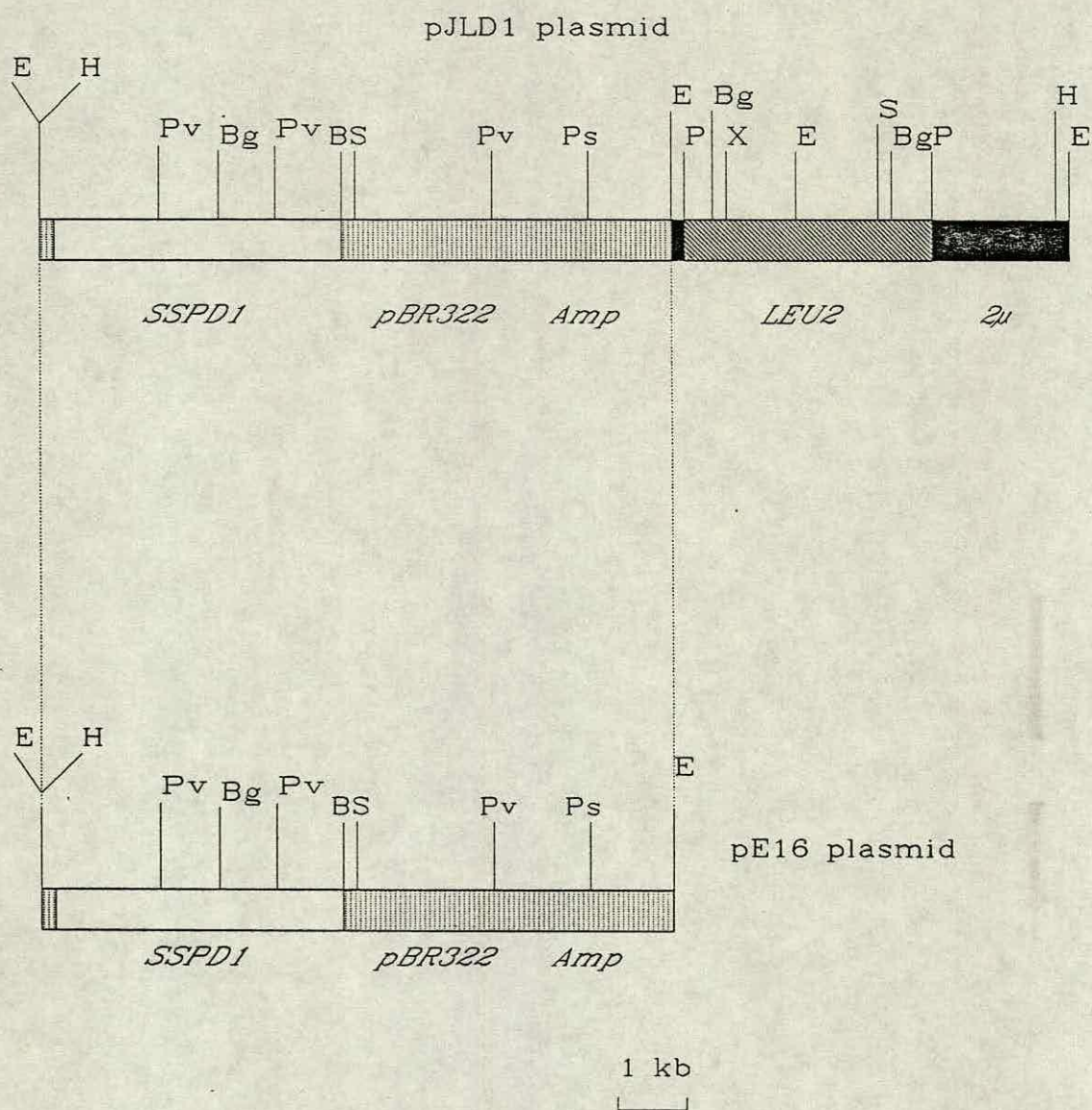


**Figure 11:** Construction of the low copy number plasmid vector pARC1. Abbreviations used are as follows: (B) *BamHI*; (E) *EcoRI*; (H) *HindIII*.







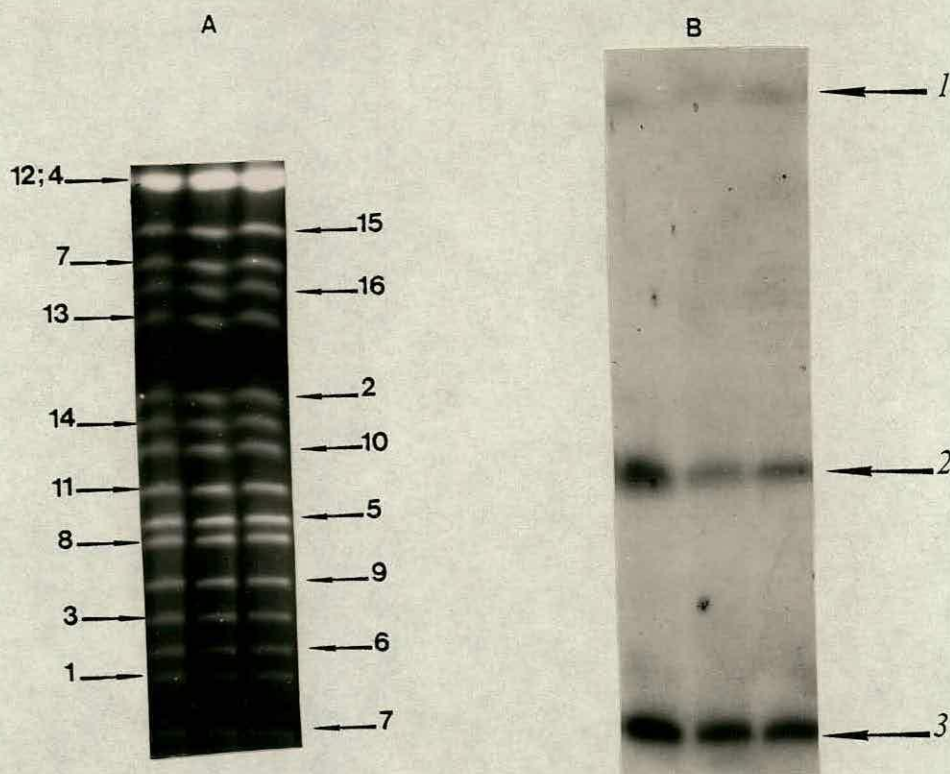


**Figure 12:** Construction of the plasmid vector pE16. All yeast sequences except *SSPD1* have been removed by *EcoRI* digestion and religation. Abbreviations used are as follows: (Bg) *Bgl*II; (B) *Bam*HI; (E) *Eco*RI; (H) *Hind*III; (Ps) *Pst*I; (Pv) *Pvu*II; (S) *Sal*I.









**Figure 13:** Chromosomal localization of the *SSPD1* sequence to chromosome VIII as shown by hybridization to separated yeast chromosomes.

(A) is a photograph of the separated chromosomes from the yeast strain YPH148 in which chromosome VII has been split in two at the *RAD2* locus (Vollrath et al, 1988).

(B) is an autoradiograph of pEI6 hybridized to the separated chromosomes. Three bands are present; bands 1 and 3 correspond to hybridization between *pBR322* sequences present in pEI6 and the engineered chromosome VII (both proximal and distal to the *RAD2* split); band 2 corresponds to hybridization between the *SSPD1* sequence of pEI6 and its chromosomal equivalent and was shown to represent chromosome VIII by comparison of migration relative to the split chromosome fragments.

Hybridization to chromosome VIII infers homology between the *SSPD1* sequence and DNA on this chromosome.







## INTEGRATIVE TRANSFORMATION USING pJIVI

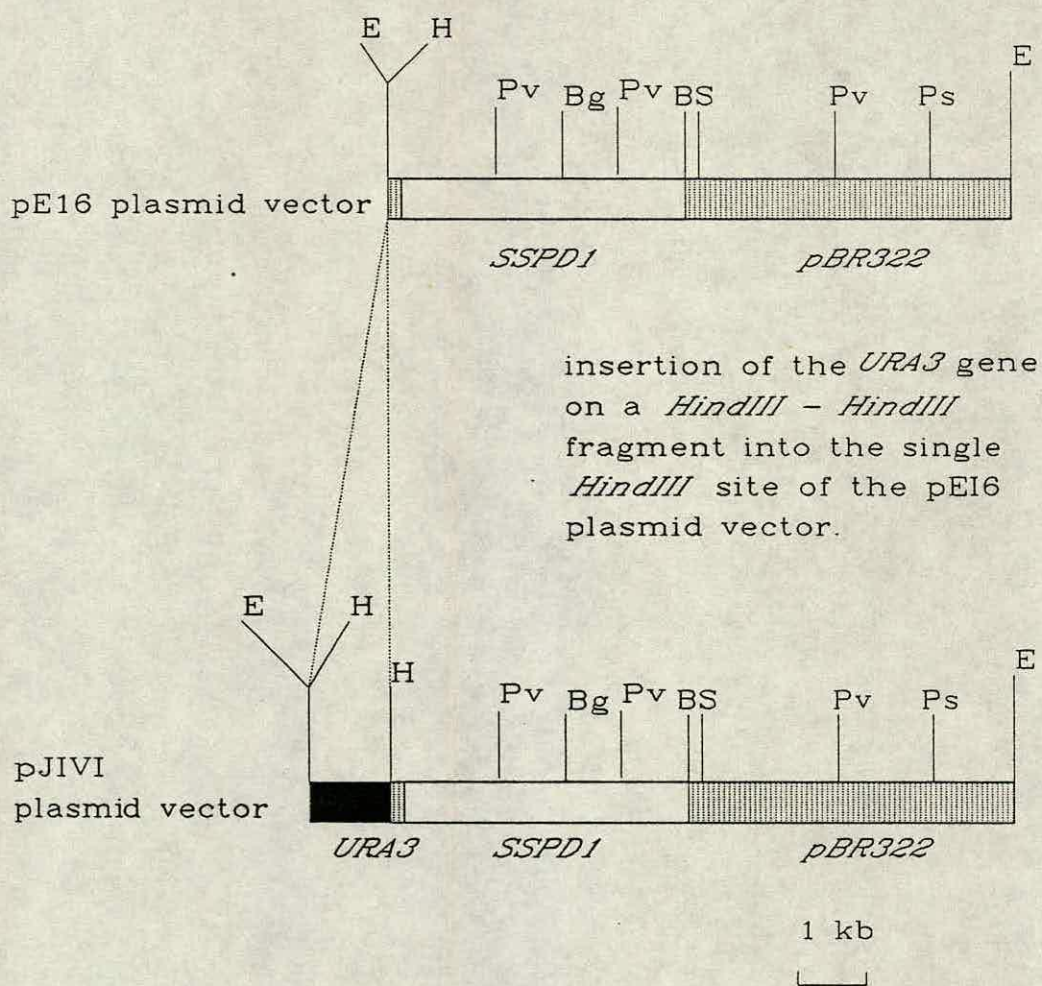
The *URA3* gene was inserted into the single *HindIII* site of pE16 to generate pJIVI, in which the only yeast sequences present are *SSPD1* and *URA3* (Fig. 14). Attempts to transform the haploid yeast strain SF747.19D to uracil prototrophy with pJIVI were unsuccessful, however transformants of the diploid strain W303 were readily obtained when cutting the sequence at a unique *BglII* site located within the complementing region of the *SSPD1* insert, thus transforming with linearized DNA.

Transformants were tested for stability by streaking individual colonies onto non-selective medium and then testing for ability to grow on uracil-DO medium. Stable transformants were sporulated and asci were dissected to separate the four spores. Half the spores were found to be uracil prototrophs and half were uracil auxotrophs suggesting that in each instance the pJIVI plasmid had integrated at a single locus. Transformants were tested genetically to determine the site of plasmid integration. One integrant, W303-INTA (*Ura*<sup>+</sup> *Spd*<sup>+</sup>) was crossed to a haploid of the strain 410:2C (*Ura*<sup>-</sup> *Spd*<sup>-</sup>) and diploid cells were selected on a medium which allowed only W303-INTA/410:2C diploids to survive. This step was necessary since the strain 410:2C is homothallic. Diploids obtained after mating and selection on minimal medium supplemented with leucine and histidine were sporulated and the resulting tetrads dissected and classified phenotypically. Eight tetrads which were dissected from this cross showed 2:2 segregation of *Ura*<sup>+</sup> : *Ura*<sup>-</sup> and *Glycerol*<sup>+</sup> : *Glycerol*<sup>-</sup> phenotypes. All combinations of phenotypes were found in approximately equal numbers (Table 5) suggesting that the site of integration of the pJIVI plasmid is not linked to the *SPD1* locus. Furthermore W303-INTA (*Ura*<sup>+</sup> *Spd*<sup>+</sup>) was crossed to a haploid of the strain 59.4A (*Ura*<sup>+</sup> *Spd*<sup>-</sup>) and diploids were selected on minimal medium supplemented with leucine and histidine. Diploids were sporulated and twelve asci dissected. Segregation of *Glycerol*<sup>+</sup> : *Glycerol*<sup>-</sup> was in the ratio 2:2 whereas *Ura*<sup>+</sup> : *Ura*<sup>-</sup> segregated in the ratios of 2:2, 4:0 or 3:1 suggesting that the pJLD1 plasmid









**Figure 14:** Construction of the integration vector pJIVI. Abbreviations used are as follows: (Bg) *Bgl*II; (B) *Bam*HI; (E) *Eco*RI; (H) *Hind*III; (Ps) *Pst*I; (Pv) *Pvu*II; (S) *Sal*I.







had integrated into a site other than the *URA3* locus.

These results, taken together with the chromosome hybridization data, imply that the *SSPD1* sequence does not contain the *SPD1* gene which has been localized to chromosome XV by genetic mapping (Dawes and Calvert, 1983).

**Table 5:** Phenotypes followed from the cross between the *S. cerevisiae* strains W303-INTA and 410:2C.

PHENOTYPE	NUMBER OF SPORES
Ura <sup>+</sup> Glycerol <sup>-</sup>	9
Ura <sup>-</sup> Glycerol <sup>-</sup>	7
Ura <sup>-</sup> Glycerol <sup>+</sup>	9
Ura <sup>+</sup> Glycerol <sup>+</sup>	7

### DISRUPTION OF THE *SSPD1* SEQUENCE

In order to test if the *SSPD1* sequence is essential for cell viability, the *SSPD1* chromosomal homologue was disrupted within the complementing region using the one-step gene disruption technique (Rothstein, 1983).

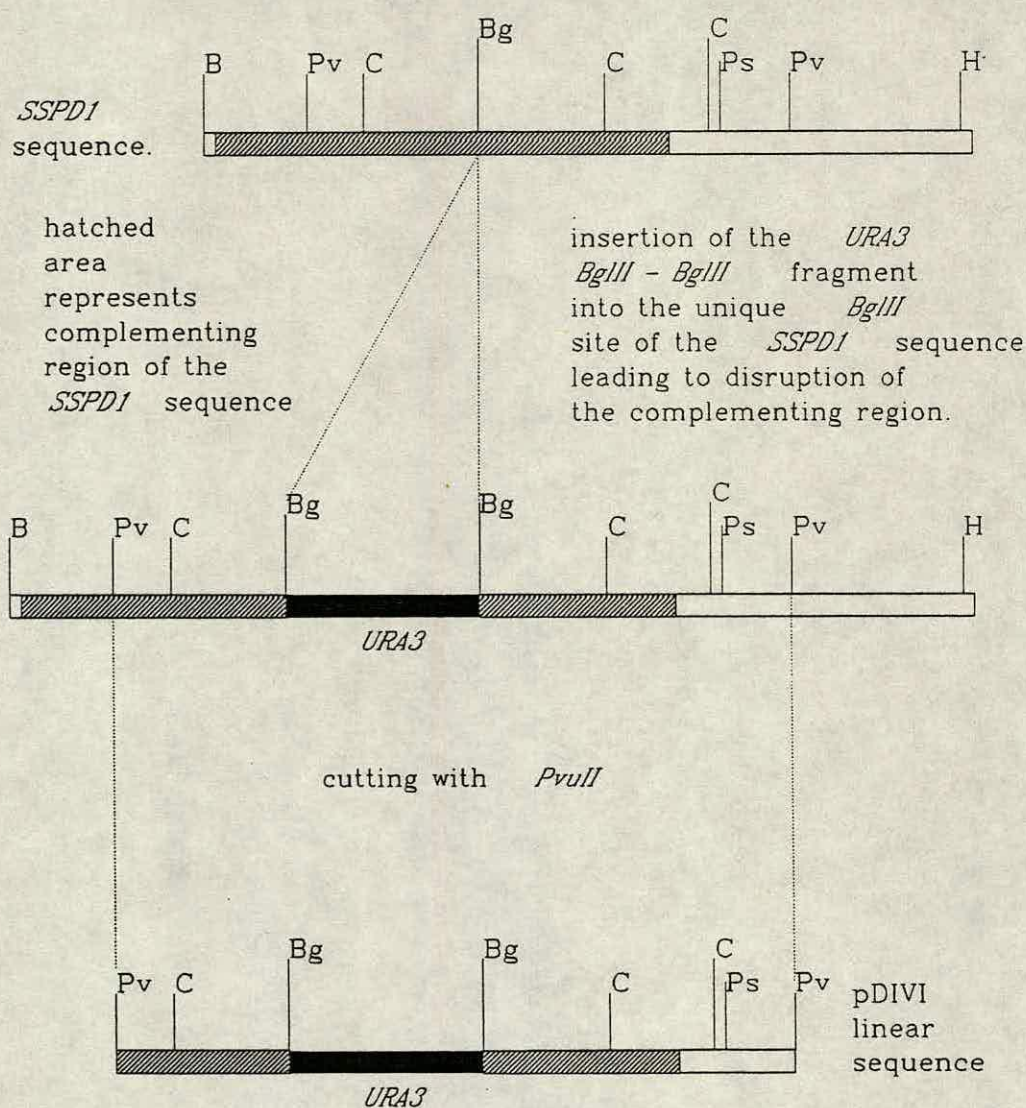
A construct was made with the *URA3* gene inserted into the *Bgl*III site of the *SSPD1* sequence, then a linear *Pvu*II-*Pvu*II fragment, named pDIVI (Figure 15), was purified using a Biotrap (see methods). pDIVI was used to transform the wild-type diploid strain W303 (Spd<sup>+</sup>, Ura<sup>-</sup>, Ade<sup>-</sup>) from uracil auxotrophy to uracil prototrophy. Figure 16 illustrates the expected recombination and integration events leading to substitution of the linear disrupted sequence (pDIVI) for the resident chromosomal sequence.

Stable Ura<sup>+</sup> transformants were sporulated and asci were dissected from 5



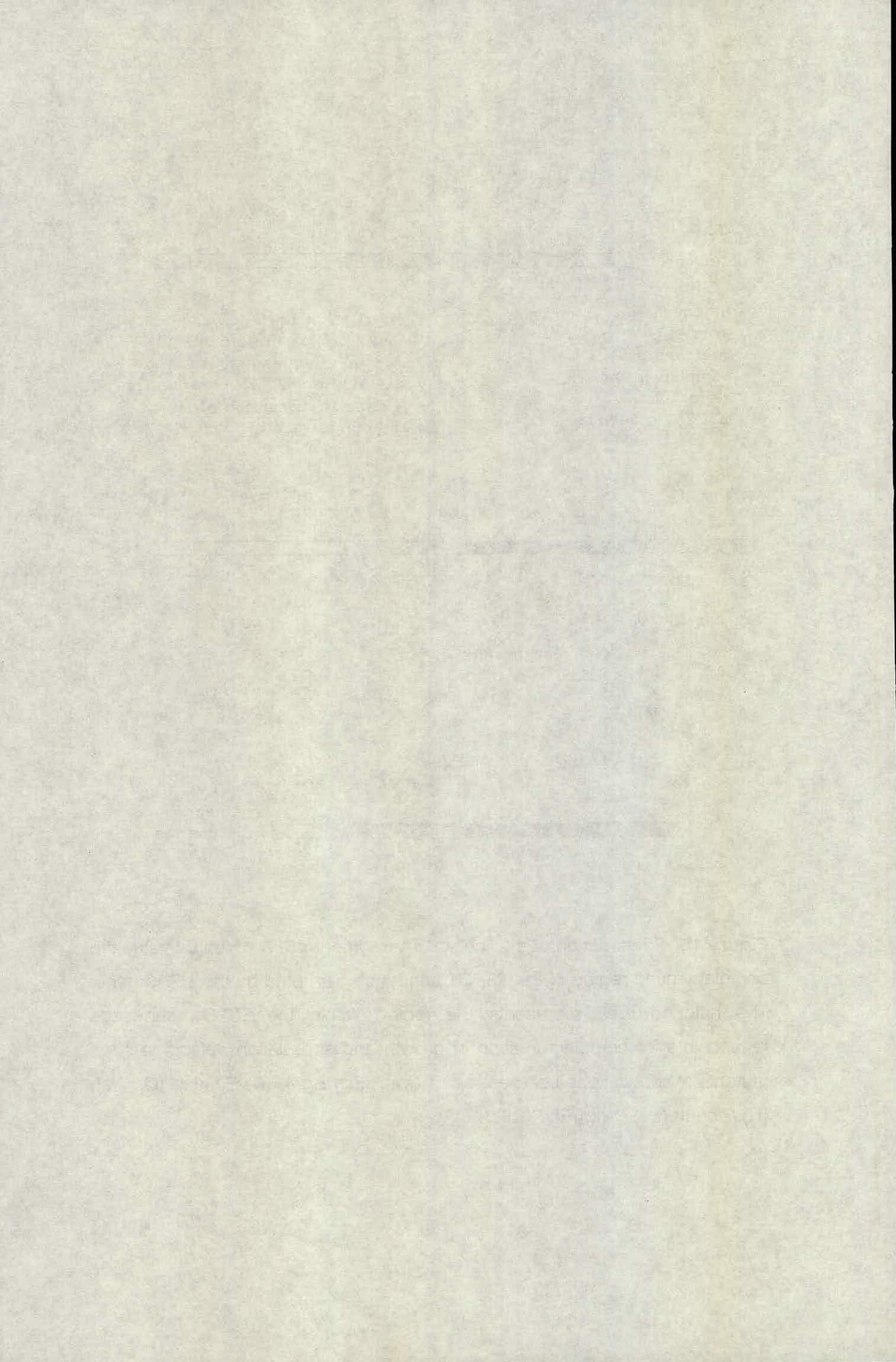




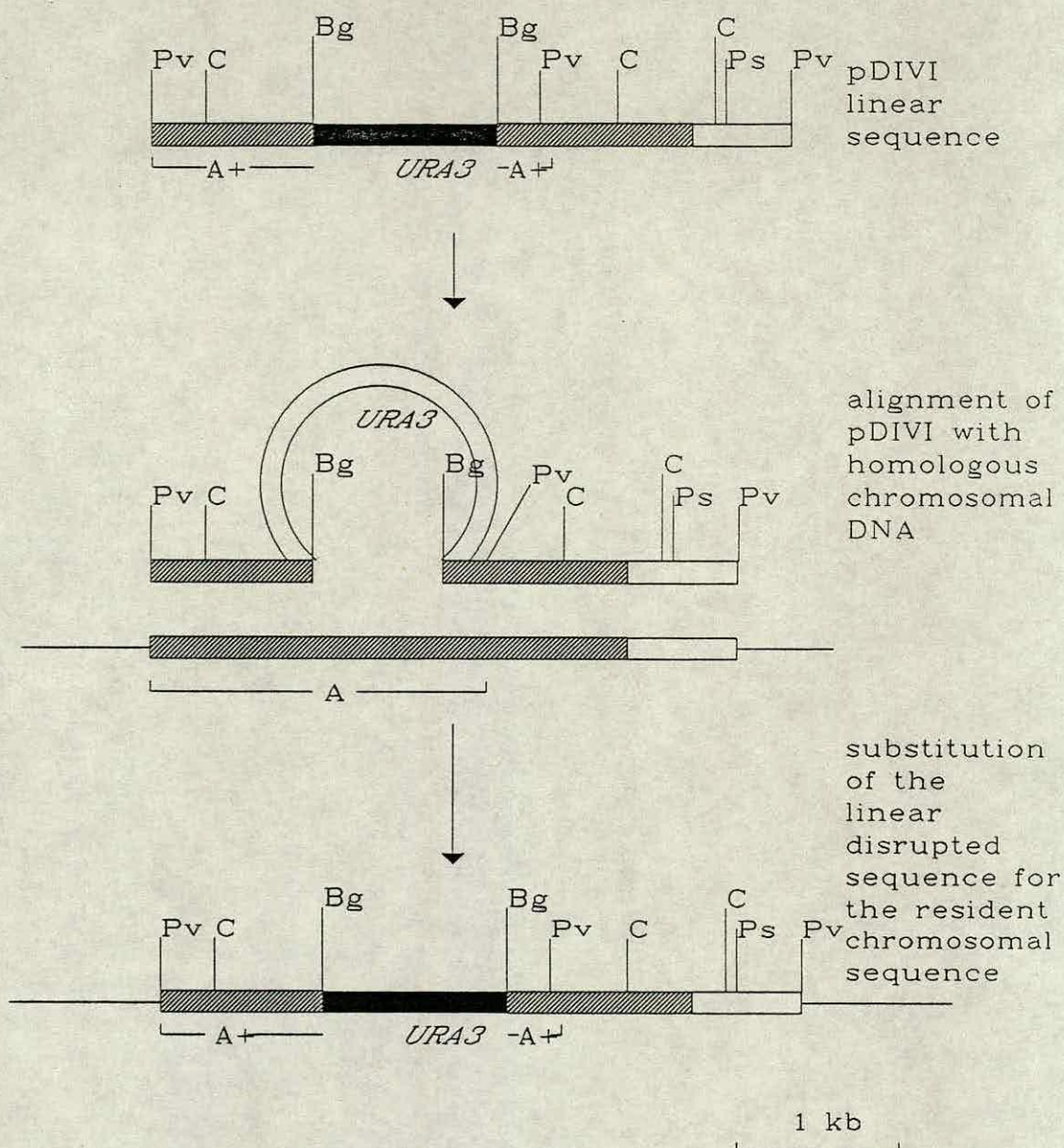


**Figure 15:** Construction of the linear DNA fragment pDIVI which contains the complementing region of the *SSPD1* sequence disrupted by the *URA3* gene. The hatched area represents the region within the *SSPD1* sequence responsible for complementation of growth and sporulation defects in *spd1* mutants. Abbreviations used are as follows: (Bg) *BglIII*; (B) *BamHI*; (C) *ClaI*; (H) *HindIII*; (Ps) *PstI*; (Pv) *PvuII*.







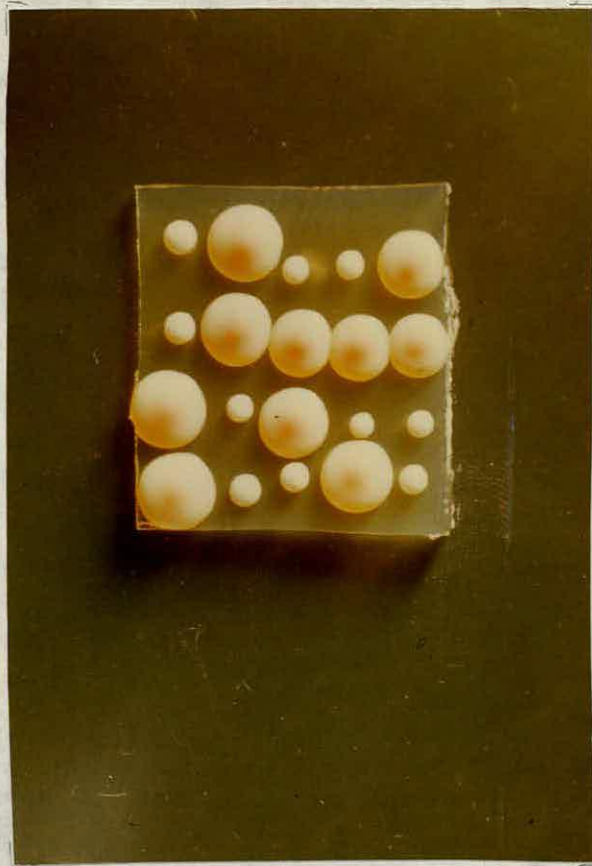


**Figure 16:** Diagrammatic representation of one-step gene disruption expected when the linear fragment pDIVI is used to transform the *Spd1*<sup>+</sup> yeast strain W303. The linear pDIVI sequence is substituted for the resident chromosomal sequence. A+ represents the complementing region of the cloned *SSPD1* sequence while A represents the chromosomal homologue of this sequence. In pDIVI the *SSPD1* complementing region has been disrupted by the selectable yeast gene *URA3*. Abbreviations used are as follows: (Bg) *BglII*; (B) *BamHI*; (C) *Clal*; (H) *HindIII*; (Ps) *PstI*; (Pv) *PvuII*.









**Figure 17:** Phenotype of cells disrupted within the complementing area of the *SSPD1* sequence i.e. at the single *Bg*/II site. A diploid heterozygous for a disruption within the complementing area of the *SSPD1* sequence (W303/W303[pDIVI]) was sporulated and asci dissected. Tetrads show 2:2 segregation of large pink to small white colonies. The photograph was taken after 4 days of incubation at 30°C. Vertical rows are spores from single asci.







different transformants. It was observed that tetrads from the 5 transformants always gave a 2:2 ratio of small, white, Ura<sup>+</sup> colonies to normal-sized, slightly pinkish, Ura<sup>-</sup> colonies (Fig. 17). These small white colonies were always Ura<sup>+</sup>. Evidence strongly supporting the view that the small white Ura<sup>+</sup> colonies were due to disruption of the complementing region of the *SSPD1* chromosomal homologue is included in the discussion. For this reason, a Southern hybridization was not carried out to verify the position of integration of the pDIVI sequence.

**Phenotype of disrupted cells.** The parent strain W303 is Ade<sup>-</sup> and appeared pink on YEPD after 48 hours, whereas the disrupted strain, W303(pDIVI), appeared white and colony size was significantly smaller than the parent. If left long enough, the disrupted strain eventually turned pink. Both strains failed to grow on adenine drop-out plates, confirming that they are both still adenine auxotrophs. The cell size of both parent and disrupted strain was not visibly different.

**Growth on complete media** containing different carbon sources was examined. It was observed that on complete media containing glucose as the carbon source (YEPD), the disrupted strain grew more slowly than the parent strain (Table 6). On complete media containing glycerol, acetate or ethanol as the sole carbon source the disrupted strain did not grow at all after 2 days at 30°C whereas at 26°C the disrupted strain grew, albeit at a significantly slower rate than the corresponding wild-type strain (Fig. 18). In a heterozygote, W303(pDIVI)/W303 the growth rate was the same as the parental strain W303/W303 suggesting that the effect of the induced mutation is recessive with respect to growth on the media tested.

**Sporulation.** Diploids were made by mixing several different pairs of W303(pDIVI) colonies on YEPD and checking with the microscope for mating structures. Individual mating structures were isolated with the aid of a light







**Table 6:** A diploid homozygous for a disruption within the *SSPD1* sequence, W303(pDIVI)/W303(pDIVI), grows significantly slower on YEPD liquid medium at 30°C than the corresponding wild-type strain. The disruption phenotype is recessive since a strain heterozygous for the disruption grows at the same rate as the wild-type. Growth was followed by measuring the optical density at 700 nm.

STRAIN	DOUBLING TIME (min) ON YEPD MEDIUM
W303/W303	66
W303/W303(pDIVI)	66
W303(pDIVI)/W303(pDIVI)	120

**Table 7:** A diploid strain disrupted in the complementing region of the *SSPD1* sequence, W303(pDIVI)/W303(pDIVI) sporulates to a significantly less extent than the corresponding wild-type diploid. The disruption phenotype is recessive since a strain heterozygous for the disruption sporulates to the same extent as the wild-type. Diploids were sporulated on YEPG media at 30°C and percentage sporulation was estimated by counting 200 cells.

STRAIN	% SPORULATION ON YEPG MEDIA AFTER 48 h
W303/W303	30
W303/W303(pDIVI)	34
W303(pDIVI)/W303(pDIVI)	5







microscope and dissection needle and the resulting diploids, W303(pDIVI)/W303(pDIVI), were tested for sporulation on various carbon sources. On complete media containing glucose, glycerol, acetate or ethanol, the diploid homozygous for the disruption sporulated to a much lesser extent than the corresponding wild-type, W303/W303, (Table 7). In a heterozygote W303(pDIVI)/W303, the level of sporulation on all media tested was the same as the parental wild-type W303/W303, indicative that the mutation is recessive for effects on sporulation on the media tested.

**Is the *SSPD1* sequence identical to the *PET1* gene located near the centromere of chromosome VIII?** Since the disruption of the *SSPD1* sequence has a *petite*-like phenotype with regards to respiration, sporulation and growth, linkage was tested between the *ARG4* gene (which is adjacent to the *PET1* locus on chromosome VIII) and the *SSPD1* sequence. The strain FD 204B (*MATa arg4 ura3*) was crossed to W303(pDIVI), the latter strain being an arginine and uracil prototroph, containing a putative disruption within the *SSPD1* chromosomal sequence by a copy of the *URA3* gene. After sporulation, 20 tetrads were dissected and segregation followed for arginine and uracil auxotrophy. For the 20 tetrads tested, free segregation was observed for the *ARG4* and *URA3* markers. These results suggest that the *SSPD1* gene is not closely linked to the *ARG4* locus and subsequently is unlikely to be identical to *PET1*.

**Reduction of TTC.** To determine whether the *SSPD1* disruption affects some aspect of mitochondrial activity, ability to reduce 2-3-5-triphenyl-tetrazolium chloride (TTC) via the respiratory chain was tested (Ogur *et al.*, 1957; Slater *et al.*, 1963). Both the parent and the disrupted *SSPD1* strain turned red with the test on YEPD indicating that both can use the respiratory pathway.







**Respiration measurements.** To test more precisely the rate of respiration in the disrupted and wild-type strains, an oxygen electrode sensor was used to measure oxygen uptake in YEPD and YEPE media. The results are shown in Table 8. The *SSPD1* disrupted strain exhibits a significantly lower rate of respiration than the wild-type strain on complete media containing either glucose (YEPD) or ethanol (YEPE) as the sole carbon source.

**Table 8:** Rates of respiration for the *SSPD1* disrupted strain, W303(pDIVI), and the corresponding wild-type strain, W303, were measured using an oxygen electrode sensor. 200ml of cells were grown to stationary phase in YEPD liquid medium at 30°C. Cells were harvested and used to produce an oxygraph for oxygen uptake in the presence of either glucose or ethanol. Measurements are given in nmoles/min/mg dry weight.

STRAIN	CARBON SOURCE	OXYGEN UPTAKE (nmoles/min/mg dw)
W303	glucose	52
W303(pDIVI)	glucose	31
W303	ethanol	51
W303(pDIVI)	ethanol	27.5

**Iodine staining.** The disrupted W303(pDIVI) strain stained brown with iodine on YEPD medium, indicative of large reserves of glycogen. The corresponding wild-type strain W303 did not stain as brown as the disruptant. These results suggest that the disruption of the *SSPD1* sequence results in a change in glycogen metabolism.







**Transformation of the disrupted strain W303(pDIVI) by the plasmid pJLD1 restores the wild-type phenotype.** The pJLD1 plasmid restores fully the growth, colour and sporulation defects of the diploid strain W303(pDIVI).

**The pJLD1 plasmid suppresses the temperature sensitive *cdc25-5* mutant.** Growth at 36°C is restored to the *cdc25-5* mutant OL97.1.4D by transformation with the pJLD1 plasmid. These results are in agreement with those of Toda *et al.* (1988).

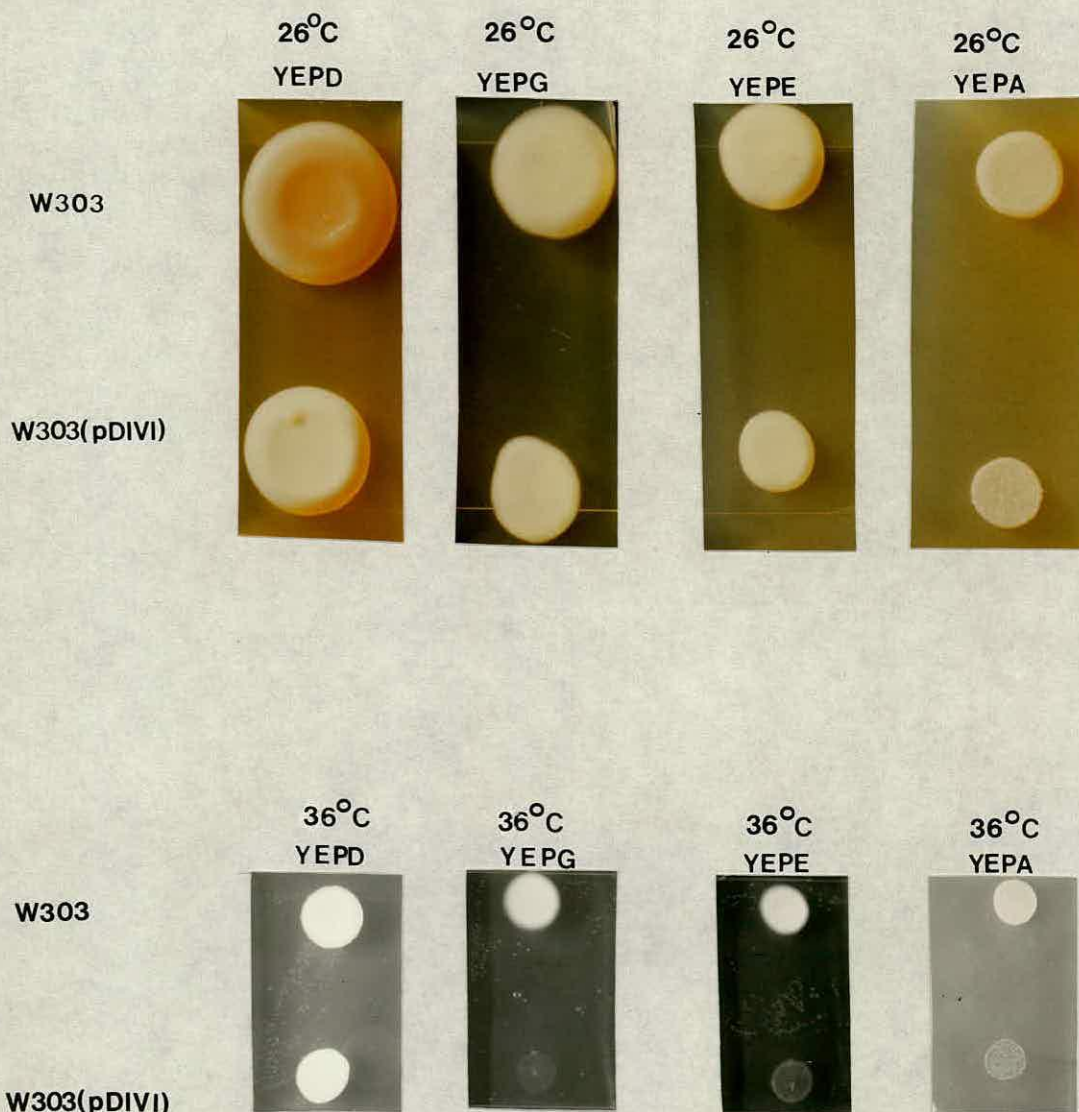
**What is the phenotype of an *spd1* mutant containing two copies of the *SSPD1* sequence?** A diploid of the homothallic strain 410:2C was transformed with the pJIVI plasmid linearized by cutting within the complementing region at the *Bgl*II site. Stable Ura<sup>+</sup> transformants were dissected and homozygous diploids formed readily when the homothallism gene *HO* was still present. These diploids grew slightly better on YEPG than did 410:2C failed to sporulate well and many abnormal shaped cells were seen to be present. These cells have a similar morphology to the *spo* mutants isolated by Calvert and Dawes (1984).

**What is the phenotype of a *sspd1 spd1* double disruptant?** The *spd1* mutant 410:2C was transformed with the pDIVI DNA fragment (Fig. 15) and stable integrative transformants were selected on uracil drop-out medium. Four tetrads were dissected on YEPD medium, each giving rise to two normally growing colonies and two colonies markedly impaired in their growth. Each of the normally growing colonies were uracil auxotrophs, suggesting that the markedly slow growth of the other dissection products was due to the disruption of the *SSPD1* sequence in an *spd1* background. It was not possible to measure the real doubling time of the *spd1 sspd1* disrupted strains due to the frequent occurrence of reversion of the *spd1* phenotype. This was probably due to the extragenic suppressor *spo* mutations (Calvert and Dawes, 1984b).



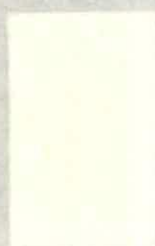






**Figure 18:** Growth of the *SSPD1* disrupted strain, W303(pDIVI), and the corresponding wild-type, W303, on solid YEPD, YEPG, YEPE and YEPA media after 3 days at 26°C and 36°C respectively. The disrupted strain grows significantly slower than the wild-type at 26°C on all media tested. At 36°C, the disrupted strain grows slower than the wild-type on YEPD and fails to grow on the media containing a non-fermentable substrate as the sole carbon source.







## SEQUENCING WITHIN THE COMPLEMENTING REGION OF *SSPD1*

To characterize further the complementing region of *SSPD1*, adjacent fragments were subcloned into the single-stranded sequencing phage M13 (Messing *et al.*, 1983) and also into the double-stranded sequencing vector PKS (provided by "STRATAGENE"). The sequence was determined by a modified version of the Sanger technique (1977). The constructs made in the sequencing vector as well as the sequencing strategy are represented in Figure 19. The PKS constructs made were;

14BCPKS which covers 1.2 kb from the *Bam*HI site at one end of *SSPD1* to the first *Cla*I site,

L1PKS which covers 0.6 kb from the first *Cla*I site to the single *Bgl*II site,

L2PKS which covers 0.8 kb from the *Bgl*II site to the second *Cla*I site and

5CCPKS which covers 1.4 kb from the first to the second *Cla*I sites.

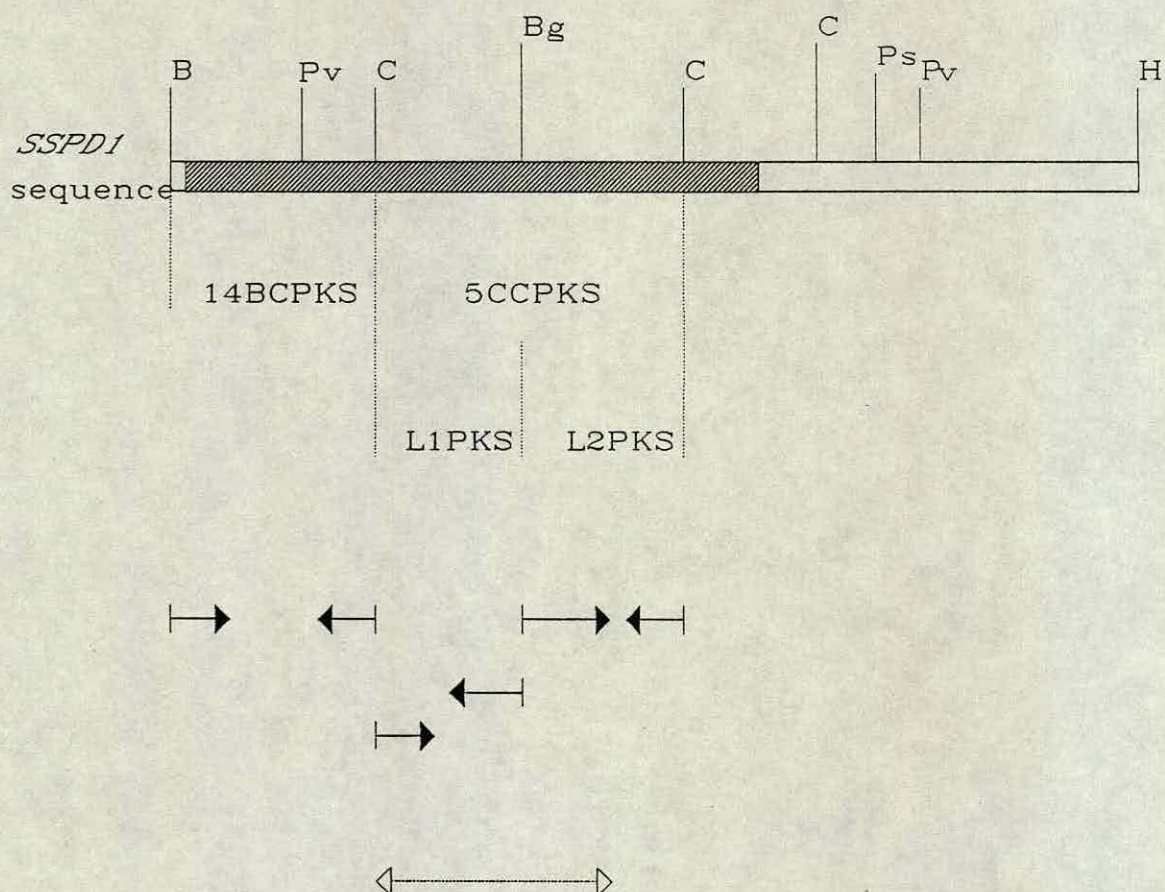
A nearly continuous fragment of 862 nucleotides was sequenced (shown by the dotted line in Figure 19) and was used in a computer search to determine whether it showed identity, at the DNA level, with any genes already isolated and sequenced. Using the Max Plank Data Bank (MIPS) information was obtained indicating 99.9% identity between the 862 nt fragment and the *SCH9* yeast gene (Toda *et al.*, 1989). Figure 20 illustrates this DNA sequence similarity. To calculate DNA sequence similarity, 60 nt missing from the sequenced fragment due to incomplete data (represented by crosses in Figure 20), were ignored. Restriction enzyme maps of the *SCH9* and *SSPD1* sequence were compared and found to be the same (Fig. 21). These results indicate that the complementing region of *SSPD1* is identical to the *SCH9* gene.

Figure 21 shows the nucleotide sequence and deduced amino acid sequence of the *SCH9* gene as reported by Toda and colleagues, (1989). The boxed area of this sequence represents the area showing 99.9% homology



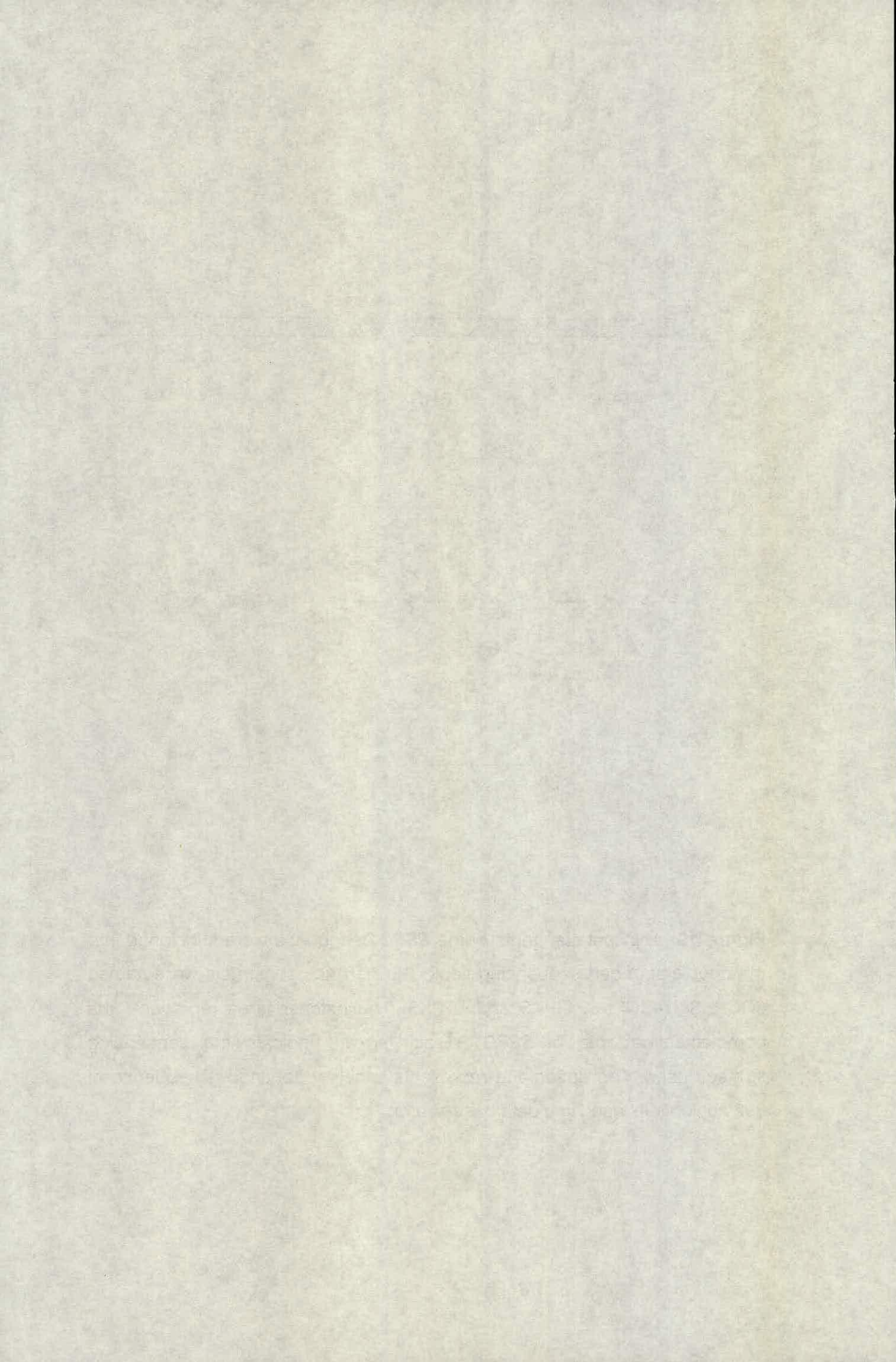






**Figure 19:** Adjacent segments of the *SSPD1* sequence were subcloned into the double-stranded sequencing vector PKS. These constructs were named 5CCPKS, 14BCPKS, LIPKS and L2PKS. The hatched area represents the complementing area of *SSPD1*. Black arrows indicate the sequencing strategy used. The dotted line represents a nearly continuous sequence of 862 nucleotides used in a data base search.







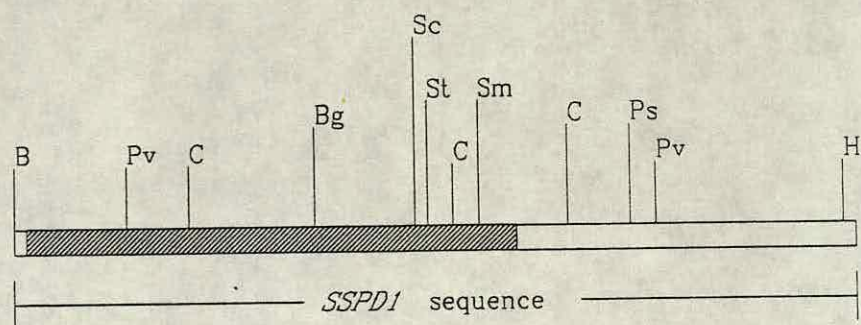
<i>SCH9</i>	CATCGATAAAAGAGCACTTGAAGAAAAAACCCCTTTATACACACAGA
<i>SSPD1</i>	CAT----AAAAGAGCACTTGAAGAAAAAACCCCTTTATACACACAGA
<i>SCH9</i>	TCATCTTCCCAATTAGATCAGCTAAACTCTTGCTCTTCAGTAACCGA
<i>SSPD1</i>	TCATCTTCCCAATTAGATCAGCTAAACTCTTGCTCTTCAGTAACCGA
<i>SCH9</i>	TCCGAGCAAACGTTCTTCTAATTCTTCGTGGGTTCTTCAAATGGTC
<i>SSPD1</i>	TCCGAGCAAACGTTCTTCTAATTCTTCGTGGGTTCTTCAAATGGTC
<i>SCH9</i>	CAAAGAATGATAGTTCACATCCAATATGGCATCACAAGACAACGTTT
<i>SSPD1</i>	CAAAGAATGATAGTTCACATCCAATATGGCATCACAAGACAACGTTT
<i>SCH9</i>	GATGTTTTGGGATCTCACTCGGAATTAGATATTTCTGTTTATGATGC
<i>SSPD1</i>	GATGTTTTGGGATCTCACTCGGAATTARATATTTCTG+++++++
<i>SCH9</i>	TGCCCACGACCATATGTTCTTAGGCCAAGTTGAAGACTGTATCCAAT
<i>SSPD1</i>	+++++
<i>SCH9</i>	GAGTCATAATTTAGCACATGCTTCCCAACACCAATGGCACAGTTTGA
<i>SSPD1</i>	+++TCATCCTTTAGCACATGCTTCCCAACACCAATGGCACAGTTTGA
<i>SCH9</i>	AACCTCGCGTTATTGATGAAGTTGTGTCCGGTGATATTTTAATCAAA
<i>SSPD1</i>	AACCTCGCGTTATTGATGAAGTTGTGTCCGGTGATATTTTAATCAAA
<i>SCH9</i>	TGGACTTACAAACAGACAAAGAAAAGACATTATGGCCCACAAGATT
<i>SSPD1</i>	TGGACTTACAAACAGACAAAGAAAAGACATTATGGCCCACAAGATT
<i>SCH9</i>	TGAAGTTCTTCGATTATTGGGTAAGGGTACTTTTGGCCAAGTCTACC
<i>SSPD1</i>	TGAAGTTCTTCGATTATTGGGTAAGGGTACTTTTGGCCAAGTCTACC
<i>SCH9</i>	AAGTTAAGAAGAAAGACACTCAAAGAATTTATGCAATGAAAGTTCTC
<i>SSPD1</i>	AAGTTAAGAAGAAAGACACTCAAAGAATTTCTGCAATGAAAGTTCTC
<i>SCH9</i>	TCCAAGAAAGTTATTGTCAAGAAAAATGAGATCGCCCACACAATTGG
<i>SSPD1</i>	TCCAAGAAAGTTATTGTCAAGAAAAATGAGATCGCCCACACAATTGG
<i>SCH9</i>	CGAAAGAAATATCCTAGTCACGACAGCGTCCAAATCGTCCCCATTCA
<i>SSPD1</i>	CGAAAGAAATATCCTAGTCACGACAGCGTCCAAATCGTCCCCATTCA
<i>SCH9</i>	TTGTCGGATTGAAGTTTTCCTTTCAAACACCAACAGATCTGTATTGG
<i>SSPD1</i>	TTGTCGGATTGAAGTTTTCCTTTCAAACACCAACAGATCTGTATTGG
<i>SCH9</i>	GTCAGTGATTATATGAGTGGTGGAGAATTATTCTGGCATTTACAAAA
<i>SSPD1</i>	GTCAGTGATTATATGAGTGGTGGAGAATTATTCTGGCATTTACAAAA
<i>SCH9</i>	GGAGGGCCGTTTTTCGGAAGACAGAGCGAAATCTATATCGCTGAGT
<i>SSPD1</i>	GGAGGGCCGTTTTTCGGAAGACAGAGCGAAATCTATATCGCTGAGT
<i>SCH9</i>	TAGTCCTAGCGTTAGAACATTTACACGATAACGATATCGTTTACAGG
<i>SSPD1</i>	TAGTCCTAGCGTTAGAACATTTACACGATAACGATATCGTTTACAGG
<i>SCH9</i>	GACCTAAAGCCTGAAAACATTCTACTCGATGCCACCGGTAACATCG <b>A</b>
<i>SSPD1</i>	GACCTAAAGCCTGAAAACATTCTACTCGATGCCAACGGTAACATCG <b>C</b>
<i>SCH9</i>	TCTTTGCGATTTTGGTCTTTCTAAAGCTGACTTGAAGGATAGAACAA
<i>SSPD1</i>	TCTTTGCGATTTTGGTCTTTCTAAAGCTGACTTGAAGGATAGAACAA
<i>SCH9</i>	ACACATTTTGGCGCACCAACGGAATACCTGGCACACAGAA
<i>SSPD1</i>	ACACATTTTGGCGCACCAACGGAATACCTGGCACACAGAA

**Figure 20:** Sequence homology between 862 nucleotides within the complementing region of the *SSPD1* sequence and the *SCH9* gene isolated by Toda *et al.* (1989). Crosses represent an area within *SSPD1* which had not been sequenced. Dashes represent the cloning site of the *SSPD1* sequence in the PKS sequencing vector. The letters in dark print represent the only base difference between the compared DNA sequence of *SCH9* and *SPPD1*. In the *SSPD1* sequence base A at position 1646 of the *SCH9* sequence was read as base C.

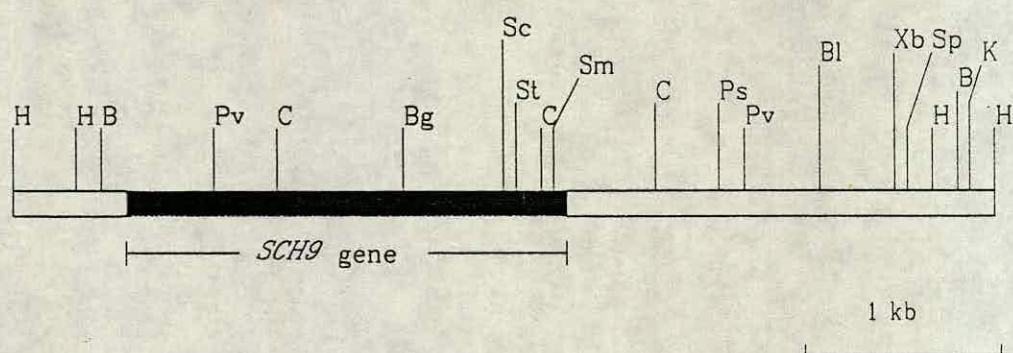








The *SSPD1* sequence. The hatched area represents the limits of the *SSPD1* sequence necessary for suppression of an *spd1* mutant for sporulation and growth on YEPG. Limits were estimated by deletion and Tn5 disruption data.



Sequence isolated by Toda *et al.* (1989). The shaded area indicates the coding sequences for the *SCH9* gene.

**Figure 21:** Comparison of restriction enzyme maps of the sequence isolated by Toda *et al.* (1989), (the shaded area indicates the coding sequences for the *SCH9* gene) and the *SSPD1* sequence. (The hatched area represents the limits of the *SSPD1* sequence necessary for suppression of an *spd1* mutant for sporulation and growth on YEPG. Limits were estimated by deletion and Tn5 disruption data). Abbreviations used are as follows: (Bg) *Bgl*II; (B) *Bam*HI; (C) *Cl*I; (H) *Hind*III; (Ps) *Pst*I; (Pv) *Pvu*II; (Sc) *Sac*I; (Sm) *Sma*I; (Sp) *Sph*I; (St) *Stu*I; (Xb) *Xba*I.







with a 862 nt fragment from the complementing region within the *SSPD1* sequence. The two sequences differ by one base at position 1646 of the *SCH9* DNA sequence (Fig 22).

The deduced amino acid sequence of the *SCH9* gene contains consensus sequences found in all of the known protein kinases (Hunter and Cooper, 1986). The sequence Gly X Gly X X Gly (where X is any amino acid), followed 7 - 16 residues later by Lys, is thought to be part of the ATP-binding site (Zoller et al., 1981; Kamps et al., 1984; Hannink and Donoghue, 1985). It is shown by inverted black triangles in Figure 22. This sequence was present within the 862 nt fragment of *SSPD1* showing 99.9% homology with the *SCH9* gene. Also present within the sequenced fragment were two other protein kinase consensus sequences: Asp Phe Gly, shown by black circles in Figure 22; and Ala Pro Glu, shown by open circles.

Less, but significant, similarity was found between the 862 nt fragment of *SSPD1* and the entire family of protein kinases (Table 9) as shown by DNA sequence comparisons.

**Table 9:** Protein kinases showing significant similarity to a 862 nt sequence from within the complementing area of *SSPD1*. Protein kinases with alignment scores greater than 30 (see Materials and Methods for details) are presented.

PROTEIN KINASE	SOURCE
Yeast PK ( <i>YKR2</i> )	Kubo <i>et al.</i> , (1989)
Yeast cAPK ( <i>TPK1</i> )	Liszewicz <i>et al.</i> , (1987)
Bovine cAPK	Shoji <i>et al.</i> , (1981)
Bovine cGPK	Takio <i>et al.</i> , (1984)
Rat PKC-type	Knopf <i>et al.</i> , (1986)







The sequence was most homologous with the yeast gene *YKR2*, yeast and mammalian cAMP-dependent protein kinases, mammalian cGPK and protein kinase C. Comparison of amino acid sequences of a portion of the *SSPD1* sequence with the protein kinases *SCH9* and *YKR2* is shown in Figure 23. Based on the results above, it is proposed that the complementing region within the *SSPD1* sequence is identical to the *SCH9* gene isolated by Toda and colleagues, (1989) and that it encodes a protein kinase.







**Figure 22:** Nucleotide sequence and deduced amino acid sequence of *SCH9* (from Toda *et al.*, 1989) are shown in the following page. The sequence data starts at the *HindIII* site at -509 bp and continues through the second *PvuII* site. In addition to the deduced amino acid sequence, 4084 nucleotides of *SCH9* are shown. The sequence in the boxed area shows 99.9% homology with a 862 nucleotide region sequenced within the complementing region of the *SSPD1* gene. Black triangles indicate the consensus sequence for amino acids at the ATP-binding site. Invariant sequences among known protein kinases are indicated (Asp Phe Gly) by black circles and (Ala Pro Glu) by white circles. The base A at position 1646 of the *SCH9* DNA sequence was read as base C in the *SSPD1* sequence.







[illegible]















## MOLECULAR CHARACTERIZATION OF THE *SSPD2* SEQUENCE







## INTRODUCTION

Since the *SSPD1* sequence isolated was shown to contain a suppressor of *spd1* and not the *SPD1* gene itself, attempts were continued to isolate the *SPD1* gene by screening *spd1* mutants with different gene banks. The same selection procedure as that used to isolate the *SSPD1* suppressor was employed here. Since there is no direct selection for the *SPD1* gene and transformants must be transferred to non-selective YEPG medium to follow sporulation, a centromeric based gene bank was used since plasmid loss is less than for a  $2\mu$  based gene bank.

## RESULTS

### SCREENING THE pSCI9-BASED GENE BANK

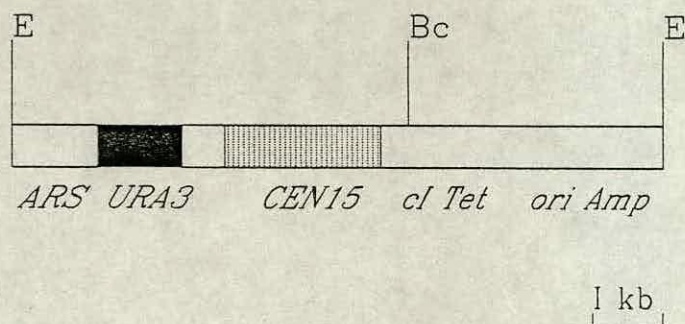
A gene bank made in the low copy plasmid vector pSC19 (Fig. 24) was kindly donated by J. Thevelein. The bank was made with a partial *Sau3A* digest of chromosomal DNA ligated to *BclI*-cut pSC19. *E. coli* transformants were selected by their ability to grow on LB plates containing tetracycline, since insertion at the *BclI* site leads to inactivation of the *cl* repressor thus allowing the tetracycline resistance gene to be expressed. The average insert of the bank was 15 kb. An *spd1* mutant, 410:2C, was transformed from uracil auxotrophy to uracil prototrophy with the bank. 5,000 Ura<sup>+</sup> colonies were picked individually to uracil-DO medium and then replica-plated onto YEPG plates. Several colonies which grew better on YEPG than the strain 410:2C were examined for the effects of plasmid loss. Eight of these colonies grew well on YEPG whether or not they contained a plasmid, indicating that these were *spo*-like mutants which had arisen spontaneously from the *spd1* strain (Calvert and Dawes, 1984). For one colony, plasmid loss corresponded to loss of growth on YEPG and corresponded also to a change in pigmentation of the cells. *spd1* strains, for unexplained reasons, are usually very white







plasmid vector  
pSC19



**Figure 24:** The plasmid vector pSC19. A pSC19-based gene bank was kindly donated by J. Thevelein. The bank was constructed by ligating partially digested *Sau3A* chromosomal DNA to the pSC19 plasmid cut at the unique *BclI* site. Average insert size in the bank was 15 kb. Abbreviations used are as follows: (E) *EcoRI*; (Bc) *BclI*.

in colour but in this particular transformant, colonies of cells containing the plasmid were a brown colour, whereas those which lost the plasmid were very white. Both the transformed and untransformed strain stained brown with iodine suggesting that both contained high glycogen reserves.

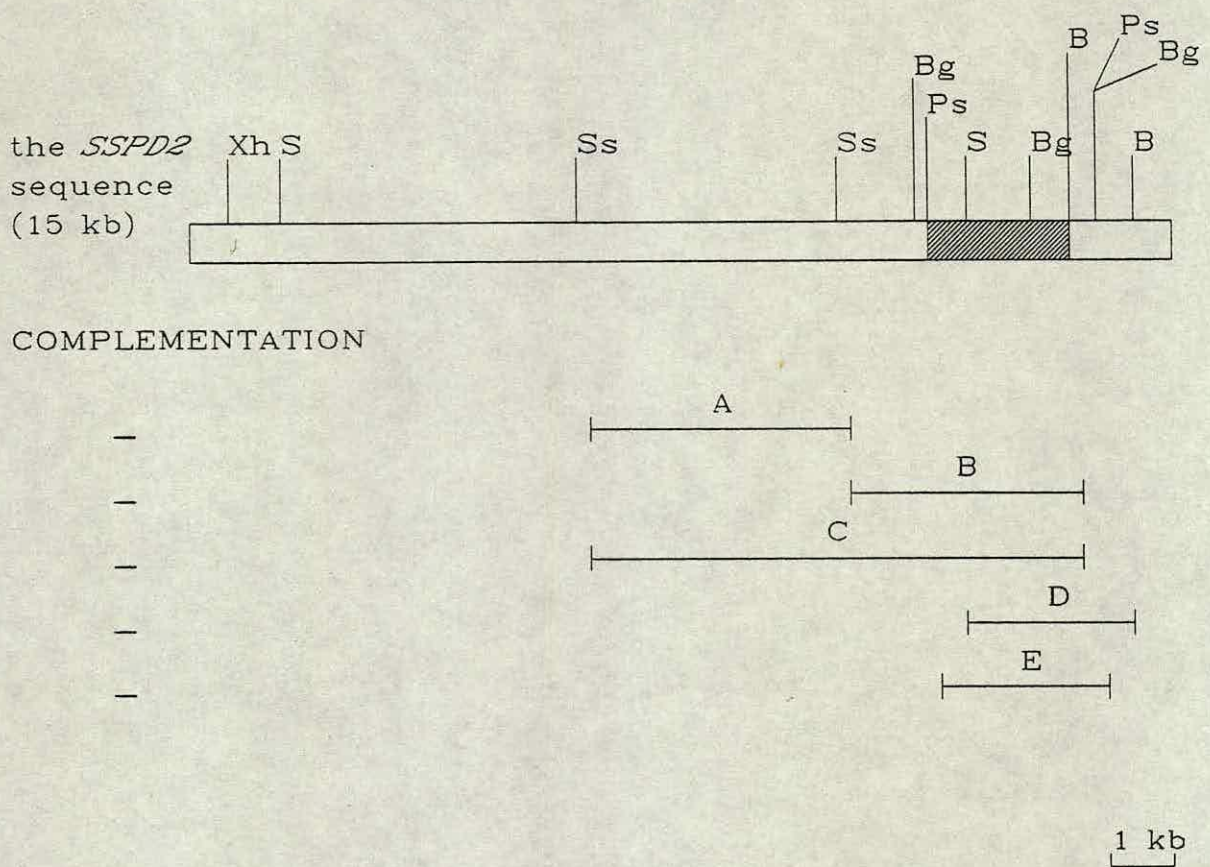
### THE SSPD2 SEQUENCE

A plasmid extraction was carried out on the brown *Ura*<sup>+</sup> colony of cells, described above, and the plasmid used to transform *E. coli* strain HB101.









**Figure 25:** Restriction map of the *SSPD2* sequence. Abbreviations used are as follows: (B) *Bam*HI; (Bg) *Bgl*II; (Ps) *Pst*I; (S) *Sal*I; (Ss) *Sst*I; (Xh) *Xho*I. Enzymes which do not cut are *Cl*aI, *Sm*aI, *Stu*I, *Sph*I and *Kpn*I. Enzymes which cut more than three times are *Hind*III, *Ava*I, *Pvu*II, *Eco*RI and *Xba*I. Horizontal lines A, B, C, D and E represent regions which have been subcloned into the plasmid vector YEp352 - none of these subclones could complement an *spd1* mutant for growth and sporulation on YEPG. The shaded area represents a *Pst*I - *Bam*HI fragment which was subcloned into the integrative vector YIp352 (Fig. 26).







Plasmid DNA from three *E. coli* transformants was isolated and each was shown to carry the same sequence of 15 kb (shown by restriction enzyme analysis). This plasmid was used to retransform the strain 410:2C and resulted in the same phenotype as before i.e. brown coloured cells with increased growth on glycerol. Microscopic examination of cells on YEPG indicated that the transformed strain sporulated to a significantly lesser extent than the corresponding untransformed strain (Table 10).

A restriction map of the *SSPD2* sequence is shown in Figure 25. Several subclonings of *SSPD2* were constructed but each of these constructs failed to complement the strain 410:2C for growth and colour change on YEPG.

### **DOES THE *SSPD2* SEQUENCE MAP CLOSE TO THE *SPD1* LOCUS?**

To test whether the *SSPD2* sequence possibly contained the *SPD1* gene, an integrative plasmid YIpS2, containing a 2.4 kb *Pst*I - *Bam*HI fragment from the *SSPD2* sequence (Fig. 26), was used to transform the wild-type strain W303 to uracil prototrophy. Prior to transformation YIpS2 was cut at the unique *Bgl*II site present within the insert. This should facilitate integration at the homologous *Bgl*II site in W303 chromosomal DNA.

Ura<sup>+</sup> transformants were tested for stability and one transformant was dissected giving rise to 2:2 segregation of Ura<sup>+</sup> : Ura<sup>-</sup> phenotypes. A Ura<sup>+</sup> colony named INTS2 was then crossed to a 410:2C haploid and resulting diploids were selected for on minimal media supplemented with leucine and histidine. Resulting diploids were sporulated and seven tetrads were dissected and classified phenotypically. Ura<sup>+</sup> : Ura<sup>-</sup> and Glycerol<sup>+</sup> : Glycerol<sup>-</sup> phenotypes segregated in a 2:2 ratio. All combinations of phenotypes were found in equal numbers suggesting that the site of integration of the YIpS2 plasmid was not closely linked to the *SPD1* locus nor to the *URA3* locus. A southern hybridization still remains to be carried out to show that the integrative plasmid integrated at the *SSPD2* site and not somewhere else in the genome.

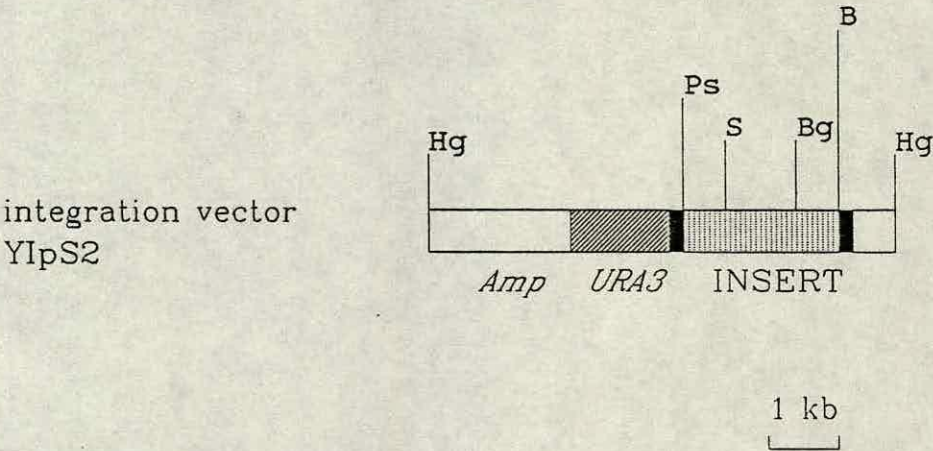






**Table 10:** Percentage sporulation of the *spd1* diploid strain 410:2C is reduced on YEPG solid medium when the strain is transformed with the *SSPD2* sequence on a centromeric plasmid (pSCAR). Cells were incubated at 30°C for 48 h and 200 cells counted for each strain.

STRAIN	% SPORULATION AFTER 48 h ON YEPG SOLID MEDIUM
410:2C	71
410:2C(pSC19)	60
410:2C(pSCAR)	24



**Figure 26:** YlpS2 an integration vector containing a 2.4 kb *PstI* - *BamHI* fragment from the *SSPD2* sequence inserted into the multiple cloning site of the integration vector Ylp352. YlpS2 was cut at the unique *BglII* site within the insert before transforming a *ura3*, *spd1* mutant. Abbreviations used are: (B) *BamHI*; (Bg) *BglII*; (Hg) *HgeII*; (Ps) *PstI*.







INTERACTIONS BETWEEN ELEMENTS OF THE cAMP SECOND  
MESSENGER PATHWAY AND THE *spd1* MUTANTS







## INTRODUCTION

In view of similarities in phenotype between the *spd* mutants and certain mutants of the cAMP signal pathway (Tatchell *et al*, 1985), and the fact that the *SSPD1* sequence (*SCH9*) suppresses mutations of the cAMP cascade (Toda *et al*, 1988) as well as suppressing the *spd1* mutation, an important question posed is:-

What is the relative position of the *spd* genes with respect to the cAMP second messenger pathway cascade?

Janice Doull (personal communication) found that the *SPD1* gene was not allelic to *CDC25*, *CDC35*, *RAS2* or *RAS1* but the question still remains whether the *SPD* genes form part of the cAMP cascade or are involved in a separate system. Interactions between elements of the cAMP second messenger pathway, the *SSPD1*, *SSPD2* sequences and an *spd1* mutant have been partially analysed. This analysis may give some indication of the relative positions of these elements with regards to each other but, as yet, is incomplete. Results are summarized in Table 11.

## RESULTS

### **AN *spd1* MUTANT TRANSFORMED WITH THE *CDC25* AND *TPK1* GENES - EFFECTS ON HYPERSPORULATION AND GROWTH ON YEPG.**

The *CDC25* gene product is the earliest component of the cAMP-protein kinase cascade of control, while the *TPK1* gene product is one of the protein kinases at the end of the specific part of the control pathway.

The *spd1* mutant strain 410:2C was transformed with the plasmid pL25.6.1 (Garreau *et al.*, 1990) which contains the *CDC25* gene inserted into pBM272 (Camonis *et al.*, 1990). The *CDC25* gene is thus under the control of the galactose inducible *GAL1-GAL10* yeast promoter. The plasmid was found to be responsible for slightly decreasing hypersporulation of *spd1* mutants on







YEPG but did not suppress the growth defect of the strain on this medium.

Transformation of the same strain with the plasmid pCB23 (kindly donated by Jacques Camonis), which contains the *TPK1* gene inserted into the centromeric vector YCp50 (Rose *et al.*, 1987) also resulted in a slight decrease in hypersporulation but no visible increase in growth on YEPG medium.

On the other hand, the strain 410:2C transformed with the plasmid pSL118 (kindly donated by Simchen), containing the *TPK1* gene inserted into the 2 $\mu$ -based vector YEp13 (Broach *et al.*, 1979) exhibited neither decreased hypersporulation nor increased growth on YEPG.

This apparent contradiction of action of the *TPK1* gene may be explained by the fact that the pSL18 plasmid is lost at a very much higher rate than pCB23 when grown on a non-selectable medium such as YEPG.

**Table 11:** Transforming an *spd1* mutant with genes from the cAMP signalling pathway - effects on hypersporulation and growth on YEPG medium.

Individual transformants were picked onto YEPG plates and incubated at 30°C for 48 h, after which sporulating cells were counted.

PLASMID	% SPORES	GROWTH ON YEPG
-	63	none
YEp13	60	none
pSCAR	25	some growth
pJLD1	30	some growth
pGAL 25.6.1	40	none
pCB23	35	none
pSL18	56	none
pRYC	41	none
pHY	32	none
YpRG1	29	some growth
YpRG3	35	none







## **THE *SSPD1* GENE SUPPRESSES THE TEMPERATURE SENSITIVE *cdc25-5* ALLELE BUT THE *SSPD2* GENE DOES NOT.**

Since the *SSPD1* and *SSPD2* are suppressors of *spd1* mutants an analysis of their relationship with the cAMP pathway may give an insight into any interactions between the *SPD1* gene and elements of the cAMP gene cascade.

In agreement with the results of Toda *et al.*, (1988) the *SSPD1* gene (*SCH9*) suppresses the *cdc25* temperature-sensitive mutation when in multicopy. It was found however, that the *SSPD2* gene did not suppress the mutation although this sequence was tested only on a centromeric vector. To test the effect of the *SSPD*" sequence in a multicopy vector will require determining the location of the essential region of the available plasmids.

## **THE *RAS<sup>ile152</sup>* MUTANT GENE CONFERS A 'DEREGULATED' ADENYLATE CYCLASE PHENOTYPE TO AN *spd1* MUTANT**

The *RAS<sup>val19</sup>* and *RAS<sup>ile152</sup>* mutant genes both bypass the *CDC25* requirement for the activation of adenylate cyclase in *S. cerevisiae* resulting in deregulation of the cAMP cascade (Broek *et al.*, 1987; Camonis and Jacquet, 1988). The *RAS<sup>ile152</sup>* gene, inserted into YCp50 and YEpl352 vectors, was used to transform the *spd1* mutant 410:2C (Table 11). Transformants had a typical 'deregulated' phenotype which resulted in low glycogen levels, decreased sporulation and abnormally shaped cells; furthermore, transformants did not grow on YEPG medium.







## THE C-TERMINAL DOMAIN OF THE *SCD25* GENE SUPPRESSES HYPERSPORULATION AND THE GROWTH DEFECT OF *SPD1* MUTANTS ON YEPG MEDIUM.

*SCD25* is a gene whose C-terminal domain was isolated as a suppressor of the *cdc25-5* mutation. The entire gene does not suppress the *cdc25* mutation leading to the hypothesis that *SCD25* normally has a target other than *CDC25* and that the specificity of *SCD25* is coded by the N-terminal part of the gene (Boy-Marcotte, *et al.*, 1989). The specific role of this gene in cells has yet to be elucidated although it is postulated that it may be a GTP-GDP exchange factor like *CDC25*, but acting on a cAMP-independent gene cascade.

*SCD25* was used to transform the *spd1* mutant but failed to suppress either the hypersporulation or growth defects of the mutant on YEPG. The C-terminal part however, decreased sporulation and increased growth of the *spd1* mutant on YEPG but led to the formation of abnormally shaped cells. Thus the *SCD25*-C terminal, which may possess GTP-GDP exchange activity, seems to suppress the *spd1* mutation.







## DISCUSSION







## THE *SSPD1* SEQUENCE CONTAINS THE *SCH9* GENE WHICH CODES FOR A PROTEIN KINASE

A region of about 1.7 - 2.6 kb within the *SSPD1* sequence is responsible for suppressing growth and sporulation defects of *spd1* mutants on YEPG medium. 862 nucleotides sequenced from within this complementing area showed 99.9% sequence homology with a region of the *SCH9* gene. The complete restriction map of *SCH9* corresponds to a 2.5 kb region within the complementing area of the *SSPD1* sequence. From these findings it can be concluded that the complementing area within the *SSPD1* sequence corresponds to the *SCH9* gene. *SCH9* has been shown to be a gene encoding a protein kinase and was isolated as a suppressor of the *cdc25<sup>ts</sup>* mutation (Toda *et al.*, 1988). Sequencing showed the presence of a long open reading frame (ORF). If translation starts at the first ATG codon in the ORF, the gene would encode a 90,000-dalton polypeptide. The deduced amino acid sequence of the *SCH9* gene contains consensus sequences found in all of the known protein kinases (Hunter and Cooper, 1986). The sequence Gly X Gly XX Gly (where X is any amino acid), followed 7-16 residues later by Lys, is thought to be part of the ATP-binding site (Zoller *et al.*, 1981; Kamps *et al.*, 1984, Hannink & Donoghue, 1985). Downstream of the ATP-binding site, two other protein kinase consensus sequences are found: Asp Phe Gly, and Ala Pro Glu, (Fig. 22). After a computer search of GenBank and the NBRF (PIR) data base, it became clear that there was significant homology between *SCH9* and the entire family of protein kinases. The *SCH9* protein kinase was most homologous to the catalytic subunits of the cAMP-dependent protein kinases (*TPK* proteins) from both yeast and mammals. (Fig.23). In fact, the putative catalytic domain of *SCH9* is closer to the cAPK catalytic domains than any other protein kinase encountered. *SCH9* also has lower but significant homologies with the catalytic domains of mammalian protein kinase C and cGPK. Like protein kinase C and cGPK, but







unlike cAPK catalytic subunits, the SCH9 protein has a large amino-terminal domain. It is presumed that this domain is regulatory, but no homology has been found between it and the regulatory domains of other protein kinases. The SCH9 protein also has homology with the YRK2 protein kinase of yeast; the *YRK2* gene was isolated by hybridization using cloned cDNA for rabbit protein kinase C as a probe (Kubo *et al.*, 1989).

## **ROLE OF PROTEIN KINASES**

The discovery that the *SSPD1* sequence is identical to the *SCH9* gene, a protein kinase, is very interesting. Protein kinases play a central role in the regulation of metabolism, in both yeast and higher eukaryotes.

Extracellular signals are transmitted to the interior of a cell probably activating transmembrane signalling systems that control production of a relatively small number of chemical mediators or second messengers such as cAMP or PIP<sub>2</sub>. These regulate protein kinase and phosphatase activities and so alter the phosphorylation states of many intracellular proteins. Protein kinases may phosphorylate seryl, threonyl, or occasionally tyrosyl residues, triggering conformational changes in the protein substrates. This alters their biological properties resulting in a change in their activities. Phosphorylation of an enzyme frequently changes the  $K_m$  for a substrate, the  $K_a$  for an activator or the  $K_i$  for an inhibitor. Substrates, activators and inhibitors may also affect the rate at which an enzyme is phosphorylated or dephosphorylated. Physiological substrates for protein kinases contain consensus sites of phosphorylation and additionally the primary structure of a protein may also be involved in protein kinase recognition.

### **What are the substrates of protein kinases?**

The number of phosphorylation and dephosphorylation reactions in a cell may appear to make the control of metabolism sound very complex indeed,







however in general the enzymes involved in biodegradative pathways are usually activated by phosphorylation: e.g. in yeast, trehalase is activated by cAMP-dependent protein kinase (Matsumoto *et al.*, 1983) whereas most enzymes involved in biosynthetic processes are inactivated by phosphorylation and reactivated by dephosphorylation: e.g. gluconeogenic enzymes such as fructose 1,6-biphosphatase, cytoplasmic dehydrogenase and PEP-carboxykinase are inactivated by phosphorylation of cAMP-dependent protein kinase in yeast. NAD-dependent glutamate dehydrogenase is also inactivated by phosphorylation by protein kinases. (Uno *et al.*, 1984).

### **INTERACTION OF THE *SCH9* GENE WITH THE cAMP SIGNAL PATHWAY**

Toda and colleagues (1988) showed that multicopy plasmids containing the *SCH9* gene suppress the growth defects of strains lacking either *CDC25*, the two *RAS* genes, the *CDC35* gene which encodes adenylate cyclase, and the three *TPK* genes, which encode the cAMP-dependent protein kinase catalytic subunits. Small colony size, resulting from disruption of the *SCH9* gene, is suppressed by activation of the cAMP pathway e.g. disrupted *BCY* function. *RAS<sup>val19</sup>* also suppresses the effect of the *sch9* mutation on conferring a growth defect on glucose. This was interpreted as being evidence that the *SCH9* gene product has functions that overlap those of the yeast cAMP pathway (Toda *et al.*, 1988) and that *SCH9* may encode an effector kinase for a growth regulatory pathway, which is, to a large extent redundant with the cAMP pathway. Another interpretation can be made - that *SCH9* is involved in a quite separate signalling pathway and that its kinase has sufficient activity when overexpressed to partially replace the cAMP-dependent protein kinase. The latter explanation is more likely in view of the extensive effect on growth rate of disrupting the *SCH9* gene as shown here and by Toda *et al.*, (1988). Further effects of the *SCH9* disruption are discussed below.







## **INTERACTION OF *SCH9* WITH THE SPORULATION MUTANT *spd1***

*SCH9* was also isolated by J. Doull (as the *SSPD1* sequence) by its ability in multicopy to restore growth of *spd1* mutants on YEPG medium. The gene also suppresses hypersporulation of *spd1* mutants on YEPG medium and turns colonies brown in colour. On a low-copy plasmid vector the gene does not give rise to any of the above effects and the *SCH9* gene is not *SPD1*. One interpretation of these results is that *SPD1* encodes a related protein kinase and that this can be replaced in part by the *SCH9* encoded protein kinase but not by the *TPK1*-encoded protein kinase. Another interpretation is that the *SPD1* gene encodes a signal pathway enzyme that is not a protein kinase but which regulates the activity of a protein kinase and that, perhaps, *SCH9* encodes the protein kinase in this pathway.

## **INTERACTIONS BETWEEN THE cAMP PATHWAY GENES AND THE *spd1* MUTANTS**

Various genes from the cAMP signalling pathway were used to transform the *spd1* mutant strain 410:2C (Table 11). The *CDC25* and *TPK1* genes when present on the centromeric vector YCp50 both reduce sporulation of the *spd1* mutant on YEPG but do not suppress the mutant's growth defect on this medium. These results suggest there may be interaction between the two pathways with regards to control of sporulation but not for growth on glycerol. Alternatively, the proteins in the cell that can be phosphorylated may have overlapping specificities as substrates for the various protein kinases e.g. some proteins involved in growth on YEPG may not be phosphorylated by *TPK1* as easily as those involved in sporulation.

Since neither *TPK1* nor *CDC25* restore growth of the *spd1* strain 410:2C on YEPG, it is unlikely that the *SPD1* gene is situated above these genes in the cAMP control cascade. Since the *TPK1* gene encodes one of the kinases at the end of this cascade then the *SPD1* gene is not a direct part of the cAMP







control system. It therefore represents another pathway or could be a gene involved in dephosphorylation of proteins.

Transformation of the *spd1* mutant 410:2C with the *RAS2<sup>ile152</sup>* (Camonis and Jacquet, 1988) gene on both centromeric and 2 $\mu$ -based plasmids resulted in a typical 'deregulated' adenylate cyclase phenotype of the cells. Cells had low glycogen levels, failed to sporulate well and did not grow on YEPG medium. These results show that the *SPD1* gene function is not likely to be involved in dephosphorylation since if it were it would suppress the *RAS<sup>ile152</sup>* phenotype. Taken with the above discussion this indicates that the *SPD1* gene belongs to a separate control pathway where functions may overlap some of those of the cAMP pathway.

The greatest effect on the *spd1* mutant was seen with the C-terminal domain of the *SCD25* gene. On a multicopy vector, this sequence decreased hypersporulation and restored slightly the growth of an *spd1* mutant on YEPG medium. It is thought that the *SCD25* gene may have *CDC25*-like function as a GTP-GDP exchange factor in yeast (Crechet, Camonis *et al.* in press) but as yet the targets of this gene have not yet been identified. It has been proposed that the N-terminal domain of the gene confers target specificity while the C-terminal domain confers activity (Boy-Marcotte *et al.*, 1989). The fact that the C-terminal domain affects the *spd1* mutant but the entire gene does not, implies that the *spd1* gene may not be the normal substrate for the *SCD25* protein while the C-terminal domain on its own acts as a GTP-GDP exchange factor with a broad range specificity. Thus the C-terminal domain may act on the pathway in which the *SPD1* gene is found, to suppress its growth and sporulation defects.

## **DISRUPTED *SCH9* PHENOTYPES**

Both Toda *et al.* and myself disrupted the *SCH9* (*SSPD1*) gene by integration







of the *ADE8* and *URA3* genes respectively at the sole *Bgl*III site. Since we both found the same phenotype it is most likely that we had disrupted the same point in the chromosome. Since Toda and colleagues had already tested, by Southern hybridization, to verify that the point of disruption was actually in the *SCH9* sequence it was not thought necessary to repeat this experiment for the *SSPD1* sequence.

The single *Bgl*III site is located in the middle of the *SCH9*-coding sequence at the 487th a.a. This separates the consensus ATP-binding sequence, Gly X Gly X X Gly, from the consensus sequence Asp Phe Gly. Both of these regions are thought to be important for kinase activity, therefore, it would be predicted that a disruption in that region would inactivate the *SCH9* protein kinase activity (Toda *et al.*, 1988).

The results in this thesis have extended the findings of Toda and colleagues. Diploid cells homozygous for a disruption within the complementing region of the *SSPD1* sequence (*sch9*), sporulate to a noticeably lesser extent than the corresponding wild-type and grow at a significantly lower rate than the corresponding wild-type on YEPD medium at 26°C and 36°C. On complete medium containing glycerol acetate or ethanol as the sole carbon source, the disrupted strain grows at a significantly slower rate than the corresponding wild-type when incubated at 26°C, and fails to grow at all when incubated at 36°C.

Toda *et al.*, (1988), found that slow growth on glucose may be suppressed by activation of the cAMP pathway. They concluded that the gene may be involved in a growth control pathway which is at least partially redundant with the cAMP pathway. The work in this thesis shows that the *SCH9* control pathway is more stringently required for growth on non-fermentable substrates.

## **PHENOTYPE OF THE *SCH9* DISRUPTION IN AN *spd1* STRAIN**

The disruption of the *SCH9* gene in an *spd1* strain resulted in extremely slow







growth on YEPD medium at 30°C. Cells divided but at such a slow rate that it was not feasible to measure doubling time.

The fact that the double disruptant has a phenotype more severe in terms of slow growth rate than the *SCH9* disruption alone, could be explained if the two genes were protein kinases with the same or an overlapping set of substrates. These results tend to exclude the possibility that the *SPD1* gene may be an element other than a protein kinase acting upstream of the *SCH9* gene, however, these types of epistatic relationship can be difficult to interpret if e.g. multiple protein kinases are present.

### **MODELS TO EXPLAIN THE ACTION OF THE *SPD1*, *SSPD1* (*SCH9*) AND *SSPD2* GENES.**

From the results so far obtained regarding the interactions between the *SSPD1*, *SSPD2* and *SPD1* genes, it can be postulated that these three genes may all encode protein kinases with similar functions in the cell and that a defect in the putative *SPD1* protein kinase can be suppressed by the *SSPD1* protein kinase when present in high dose in the cell or by the *SSPD2* protein kinase.

Alternatively, it is possible that these three sequences represent elements in a gene pathway which acts in parallel to or has overlapping functions with the cAMP gene cascade and that *SCH9* is a *SPD1*-dependent protein kinase. The *SSPD2* gene may be an element in this pathway that acts upstream of the *SCH9* gene. In this model, the *SCH9* protein may phosphorylate the same proteins as the cAMP-dependent protein kinases and may substitute for a defective *TPK1* protein when present in high enough dose in the cell. The fact that the *TPK1* gene cannot suppress either *spd1* or *sch9* mutations suggests that perhaps the proteins in the cell that can be phosphorylated have overlapping specificities as substrates for the various protein kinases and that the *TPK1*-encoded protein kinase cannot phosphorylate at least some of the *SCH9* substrates (e.g. those involved in growth on YEPG).







These two models, explaining possible *SSPD1*, *SSPD2* and *SPD1* gene functions, are illustrated in Figure 26. It should be noted that these two models are not inclusive and that different interpretations of the above results are possible. Furthermore, information is still missing to construct a more precise model of possible interactions of the *SPD1*, *SSPD1* and *SSPD2* encoded proteins.

## **EVIDENCE POINTING TO THE EXISTENCE OF cAMP-INDEPENDENT SIGNAL PATHWAYS IN YEAST**

It has been suggested that the SCH9 protein kinase may be involved in a signalling pathway in which the second messenger is not cAMP (Toda *et al.*, 1988). As mentioned in the introduction, there is mounting evidence that cAMP-independent signal pathways may exist for control of sporulation, glycogen metabolism and heat shock resistance in *S. cerevisiae* (Cameron *et al.*, 1988, Olempska-Beer and Freese, 1987). If there are other regulatory controls, then the machinery for these cAMP-independent pathways must exist. Evidence that such proteins exist and that other second messengers may mediate nutritional responses is discussed below.

### **cAMP-independent GTP-binding proteins**

Several GTP-binding proteins which act in different systems from the cAMP-dependent machinery have been isolated.

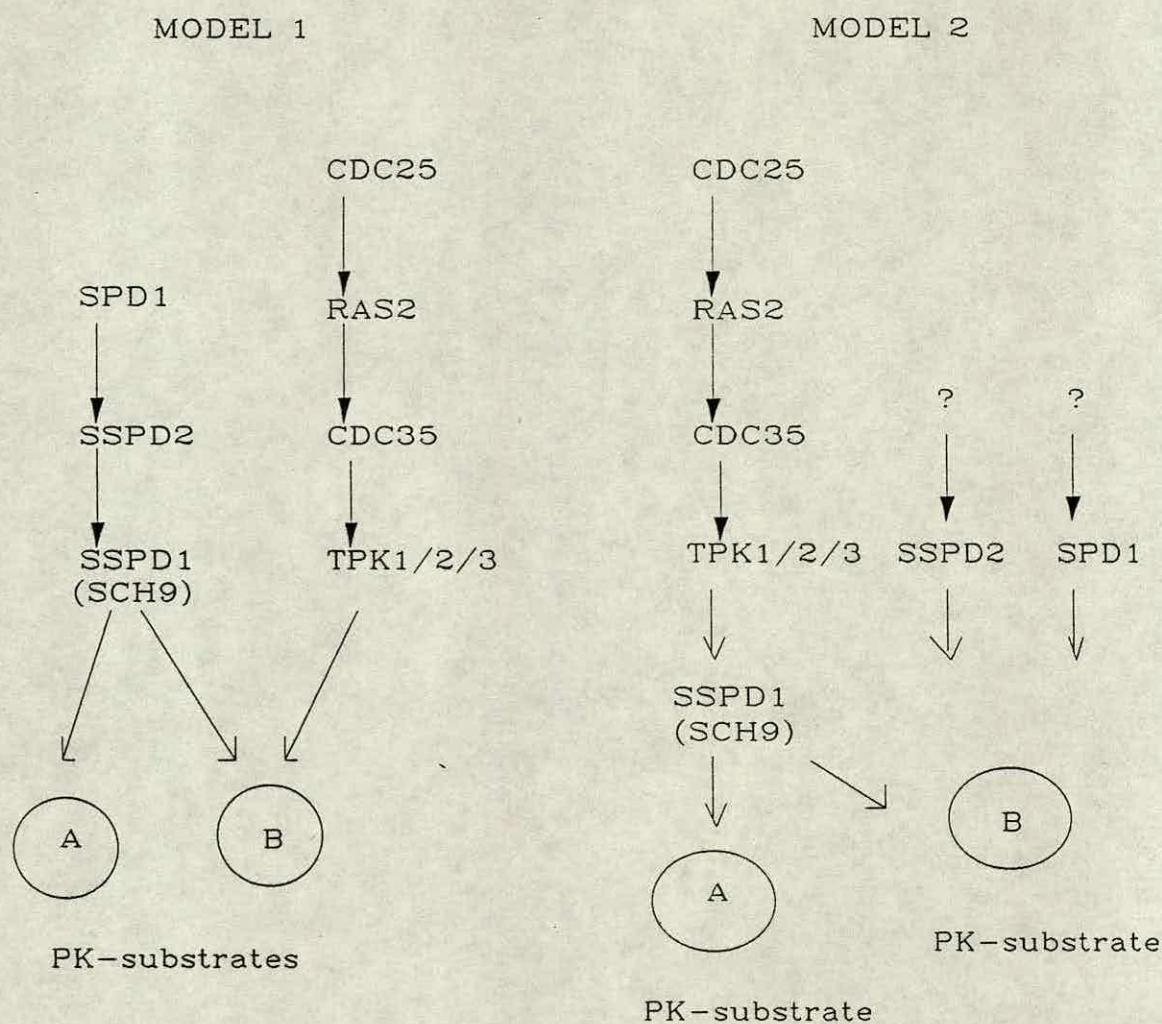
*YPT1* is a GTP-binding protein like *RAS*. It is responsible for growth, sporulation and starvation responses and is associated with the secretion machinery. There is evidence showing that a deregulation of intracellular calcium is an immediate response to a block of *YPT1* function suggesting that this guanine nucleotide-binding protein is directly involved in  $\text{Ca}^{2+}$  regulation (Schmitt *et al.*, 1988).

Other *RAS*-like proteins include the *RHO* family (Madaule *et al.*, 1987). *RHO1*









**Figure 27:** Two models depicting possible interactions between the *SPD1*, *SSPD1* (*SCH9*) and *SSPD2* genes with each other and with elements of the cAMP pathway. These models are not inclusive and the information obtained with respect to these genes does not, as yet, allow a more defined description of the roles of *SPD1*, *SSPD1* and *SSPD2* genes in the cell.







is required for cell viability while *RHO2* is not an essential gene. A mutant allele of *RHO1* analogous to a *RAS* transforming mutant has been isolated; these cells cannot sporulate. The requirement for *RHO1* cannot be circumvented by high copy numbers of cAMP-dependent protein kinase catalytic unit. *RHO1* is therefore thought to function independently of the cAMP cascade. It has been proposed that perhaps *RAS* and *RHO* proteins could link the same surface receptor to different intracellular receptors.

### **cAMP-independent guanyl nucleotide exchange factors**

GTP-binding proteins involved in cAMP-independent signalling pathways may have activity controlled by *CDC25*-like guanyl nucleotide exchange factors. *SCD25* is a gene whose C-terminal part shows 45% amino acid conservation with the C-terminal of *CDC25* (Boy-Marcotte *et al.*, 1989) and in *E. coli* the *SCD25* C-terminal domain greatly enhances the dissociation rate of GDP-bound, and to a lesser extent, of GTP-bound forms of *RAS2* proteins and of P21c-Ha-Ras. *SCD25* is therefore a possible candidate for a guanyl nucleotide releasing factor towards GTP/GDP-binding proteins, acting in a cAMP-independent signalling pathway.

### **cAMP-independent protein kinases**

The *SCH9* gene is most homologous to the catalytic subunits of the cAMP-dependent protein kinases from both yeast and mammals. In fact, the putative catalytic domain of *SCH9* is closer to the cAPK domains than any other protein kinase encountered, but unlike cAPK, the *SCH9* protein has a large amino-terminal domain. The proteins encoding the catalytic domains of the cAMP-dependent protein kinases are encoded by the genes *TPK1*, *TPK2*, and *TPK3*.







*SCH9* also shows homology with mammalian protein kinase C, (PKC) which likewise has a large amino-terminal domain. There is no homology of this domain with that of PKC but it is presumed to have a regulatory function. In mammals, PKC is activated by receptor-mediated hydrolysis of inositol phospholipids. In yeast, it has recently been shown that signal-mediated hydrolysis of inositol phospholipids in *Saccharomyces cerevisiae* generates diacylglycerol in a similar manner to that seen in the  $\text{PIP}_2$  mediated signalling pathway in mammals (Kaibuchi *et al.*, 1987). Furthermore, PKC activity has been demonstrated in *S. cerevisiae* (Levin *et al.*, personal communication) and PKC-like protein kinases have been identified (Levin *et al.*, personal communication, Kubo *et al.*, 1989)

The *SCH9* protein also shows significant homology to the bovine cGMP protein kinase (GPK) (Takio *et al.*, 1984). A yeast GPK has not yet been identified.

### **cAMP-independent signalling pathways**

In mammals, most tissues seem to have at least 2 major receptor classes for transducing information across the cell membrane (Cohen, 1985). One class depends on the generation of cAMP as the second messenger (Schramm and Selinger, 1984), while the other class induces rapid turnover of inositol phospholipids as well as mobilization of  $\text{Ca}^{2+}$  (Berridge and Irvine, 1984). The calcium mobilized in this way activates calmodulin-dependent enzymes which transmit effects of calcium ion concentration.  $\text{Ca}^{2+}$ -calmodulin alters phosphorylation states of intracellular proteins acting through calmodulin-dependent protein kinases.

In yeast the calmodulin gene has been identified and disruption or deletion of the gene results in a recessive lethal mutation; thus, calmodulin is essential for growth of yeast cells. Several lines of investigation suggest that  $\text{Ca}^{2+}$  is an important regulatory ion in yeast (for Review, see Davis and Thorner, 1986) although the role that calmodulin may play in mediating effects of  $\text{Ca}^{2+}$







in yeast is not yet known.

Kaibuchi and colleagues (1986) looked at inositolphospholipid turnover in *S. cerevisiae* and found that glucose stimulates inositolphospholipid turnover,  $\text{Ca}^{2+}$  mobilisation, and subsequent cell proliferation in a manner similar to that of growth factors in mammalian cells. In yeast, glucose is responsible for induction of a cAMP signal (Mbonyi *et al.*, 1988) and also acts to stimulate inositolphospholipid turnover, resulting in formation of inositol phosphates (Kaibuchi *et al.*, 1986).

In mammalian cells, inositolphospholipid turnover is linked to activation of protein kinase C and  $\text{Ca}^{2+}$  mobilization. In yeast, protein kinase C activity has been detected (Levin *et al.*, personal communication) and several genes showing significant homology to mammalian protein kinase C have been isolated (Levin *et al.*, personal communication, Kubo *et al.*, 1989).

In mammals the cAMP and  $\text{Ca}^{2+}$  second messenger systems are closely interlinked allowing them to act synergistically or antagonistically (Cohen, 1985). The existence of a calmodulin-dependent adenylate cyclase and cAMP phosphodiesterase implies that the level of cAMP can be regulated by  $\text{Ca}^{2+}$ . Conversely, the level of  $\text{Ca}^{2+}$  can sometimes be regulated by cAMP (Tada and Unui, 1983). Evidence exists showing certain calmodulin-dependent protein kinases are targets for cAMP-protein kinases (Cohen, 1983, Nishikawa *et al.*, 1984). Furthermore, cAMP-dependent and calmodulin-dependent protein kinases frequently phosphorylate the same proteins, although usually at distinct sites. Such proteins include glycogen synthase (Cohen, 1983) and tyrosine hydroxylase (McGuinness *et al.*, 1983, Woodgett *et al.*, 1984). It is possible that a similar control may exist in yeast.

#### **THE *SPD*'s AND *SCH9* COULD BE INVOLVED IN A cAMP-INDEPENDENT SECOND MESSENGER SIGNALLING PATHWAY.**

The *spd* mutants have a phenotype partially similar to the *ras* mutants (Tatchell *et al.*, 1985). Both groups fail to grow on non-fermentable carbon







sources, both hyperaccumulate the storage carbohydrate glycogen and both sporulate on rich media. The *ras2* mutants are similar in phenotype to the *spd*'s, although *spd*'s do grow well and fail to sporulate on medium containing ethanol as the carbon source. The *spd* mutations, like *ras2*, revert to glycerol utilization at a high frequency, via extragenic suppressors. The *spo* suppressors of *spd*'s share similarities to the *bcy1* mutant suppressors of the *ras2* mutation although the *spd* suppressors, unlike *bcy1*, are dominant for suppression of the gluconeogenic defect.

In view of the similarities in phenotype between *ras* and *spd* mutants (Tatchell *et al.*, 1985) and the fact that the *spd* mutants are not allelic to any of the cAMP pathway mutants (J. Doull, personal communication), it can be suggested that the *SPD* gene products are involved in a similar type of pathway as the RAS proteins, but that this pathway is not mediated by cAMP. Since the *SCH9* gene sequence shows important homology with yeast cAPK it is possible that *SCH9* is the functional equivalent of the *TPK* genes, but acting as a protein kinase catalytic subunit in a cAMP-independent pathway. It is possible that the *SCH9* protein kinase is regulated by the *SPD1* gene in a cAMP-independent second messenger pathway and that the *SPD1* gene is a GTP-binding protein.

Since the *SCH9* protein kinase suppresses both *spd1* and cAMP mutants it may also be involved in a cAMP-independent signalling pathway and this pathway may be interlinked but not dependent upon the cAMP machinery. Alternatively, the *SCH9* gene could be involved in a pathway that is not interlinked with the cAMP-dependent signalling pathway, but multiple copies of the *SCH9*-encoded protein kinase may have enough activity to phosphorylate a substrate which would not be phosphorylated by the *SCH9* gene product under normal conditions.

As discussed previously, there is mounting evidence that there are probably other signalling pathways with second messengers other than cAMP since in higher eukaryotes, several independent signalling pathways have been detected. In mammals, these pathways use different second messengers but







the pathways appear to be interlinked, working together synergistically at times while antagonistically at other times. RAS proteins have been shown to interact with the  $\text{PIP}_2$ -signalling pathway in yeast, another example of interactions between signalling pathways mediated by different second messengers.

In view of the significant homology of the *SCH9* sequence with mammalian PKC, which is activated by inositol phospholipids in mammals, it is suggested that *SCH9* may be involved in an analogous  $\text{PIP}_2$  signal pathway in yeast. Since it has been shown recently that in *Saccharomyces cerevisiae*,  $\text{PIP}_2$  may be involved in a signalling pathway too (Kaibuchi *et al.*, 1986), it is suggested that the *SCH9* protein kinase and perhaps the *SPD* gene product may be involved in this pathway.

## **EXPLANATION FOR ROLE OF SIGNALLING PATHWAYS AND SPORULATION**

Freese and colleagues (1982) found that sporulation could be induced by a partial deprivation of carbon, nitrogen or phosphate in *S. cerevisiae*, but that complete deficiencies of these nutrients prevented sporulation. It is suggested that a slow rate of gluconeogenesis is necessary for sporulation to occur and that potassium acetate may be a good sporulation medium, not necessarily because it has a specific role in sporulation but because its slow rate of uptake and gluconeogenic metabolism are optimal for sporulation to occur. Both  $\text{NH}_4^+$  and glucose may block sporulation by blocking gluconeogenesis. Glucose is a rapidly metabolized glycolytic carbon source which may prevent sporulation through its repression of gluconeogenic enzymes by protein kinase activation. Glucose also represses cell components such as mitochondrial enzymes which are needed for sporulation to occur.

It is proposed that the *cdc25*, *cdc35* and *spd* mutations causing







hypersporulation are not null mutations (the *ras2* mutation causing hypersporulation is a null mutation but is compensated for by an active *RAS1* gene) and that partial protein kinase activity is still present in these mutants. This may lead to only partial regulation of enzymes normally regulated by protein kinase activity e.g. gluconeogenic enzymes. A slow rate of gluconeogenic activity may be the signal for sporulation to occur on non-fermentable carbon sources. Disruptions in different areas of the *CDC25* gene lead to different effects on sporulation and growth (Munder *et al.*, 1988). For example, when the gene is disrupted in the central position of the gene, which probably inactivates the protein completely, this leads to normal growth on glucose but impaired growth on acetate, while diploids fail to sporulate.

The disruption of the *SCH9* gene at the *Bgl*II site should completely deactivate protein kinase activity (Toda *et al.*, 1988). Homozygous diploids do not sporulate well, don't grow well on glycerol and acetate sources and grow more slowly on glucose. If the mutation was induced at another point which did not fully disrupt gene function would hypersporulation occur?

TCA cycle mutants also affect growth on various C-sources and have sporulation defects. If a TCA cycle enzyme is inactive this may well affect gluconeogenesis leading to sporulation defects. Mutants homozygous for the *lpd1* mutation were found to be asporogenous, whereas heterozygotes *lpd1/LPD1* sporulated to a greater extent than *LPD1/LPD1* diploids on sporulation media (Dickinson *et al.*, 1986). This may be explained by assuming that some TCA functions and/or gluconeogenic activity is required for sporulation to occur; when this activity is lower than normal sporulation is easily induced, but if there is no activity at all (as in the *lpd1/lpd1* homozygotes) then sporulation cannot proceed.

These results are in accordance with Freese's idea that control of sporulation may involve a fine balance of metabolism (Freese *et al.*, 1982). In a normal cell when all nutrients are present at concentrations that enable unhindered growth, sporulation is prevented. A slight disruption of this balance in a particular direction may be caused by starvation conditions in a normal cell or







by a mutation which prevents transmission a signal telling the cell that nutrients are available (*cdc25*, *cdc35*, *ras2* temperature sensitive mutations and *spd1?*). Both of these conditions may lead to an increased tendency for a cell to sporulate. A more dramatic disruption of this balance in the same direction, resulting from complete deprivation of nutrients or a completely inactive protein kinase, prevents sporulation because the new mRNA and protein molecules needed for differentiation are not being made (*SCH9* and *CDC25* disruptions). At the other extreme, when constitutive protein kinase activity is present (e.g. in *RAS<sup>ile152</sup>* and *bcy1* mutants) gluconeogenic and other enzyme activities are inhibited even under nutritional conditions normally conducive to initiation of sporulation. Thus, these cells are sporulation deficient.

The effects of  $\text{NH}_4^+$  inhibition are difficult to interpret but utilization of non-fermentable carbon sources and  $\text{NH}_4^+$  metabolism seem to be linked in some way (Trevelyn and Harrison, 1956, Saita and Slaughter, 1984). It is attractive to speculate that the *SPD1* gene reflects  $\text{NH}_4^+$  control of metabolism by acting at the membrane of a cell to activate one or more second messenger systems via protein kinases. If there were a block in one pathway there would be a metabolic imbalance in which certain protein kinases are not activated normally. As a result, the cell would not be so sensitive to nitrogen suppression as normal cells.

Glycerol, acetate, pyruvate may activate the same pathway(s) as  $\text{NH}_4^+$  and so if their signal is not transmitted normally by second messenger pathways, the cells would not be fully prepared to metabolize the available C-sources. Appropriate protein kinases may not be activated fully, or at all, to phosphorylate the appropriate enzymes and so these substrates may not be used at all, or may be used at a rate slower than that found in normal cells. When the substrates can be used with difficulty this may lead to hypersporulation on non-fermentable carbon sources. When the carbon sources cannot be used at all, sporulation may not be able to occur. In a simplified form, sporulation needs some energy; if energy is low, sporulation







occurs; if no energy is available, sporulation cannot occur.

The role of glucose in repressing sporulation is unclear although it is probably due to a whole series of effects (Entian, 1986). It appears to inhibit TCA-cycle enzymes required for acetate metabolism (Miyake *et al.*, 1971) perhaps through inhibition of gluconeogenic enzymes by protein kinase phosphorylation. Glucose also represses cell components such as mitochondrial enzymes which are needed for sporulation to occur. At present, however, the precise role of glucose in sporulation is unclear.







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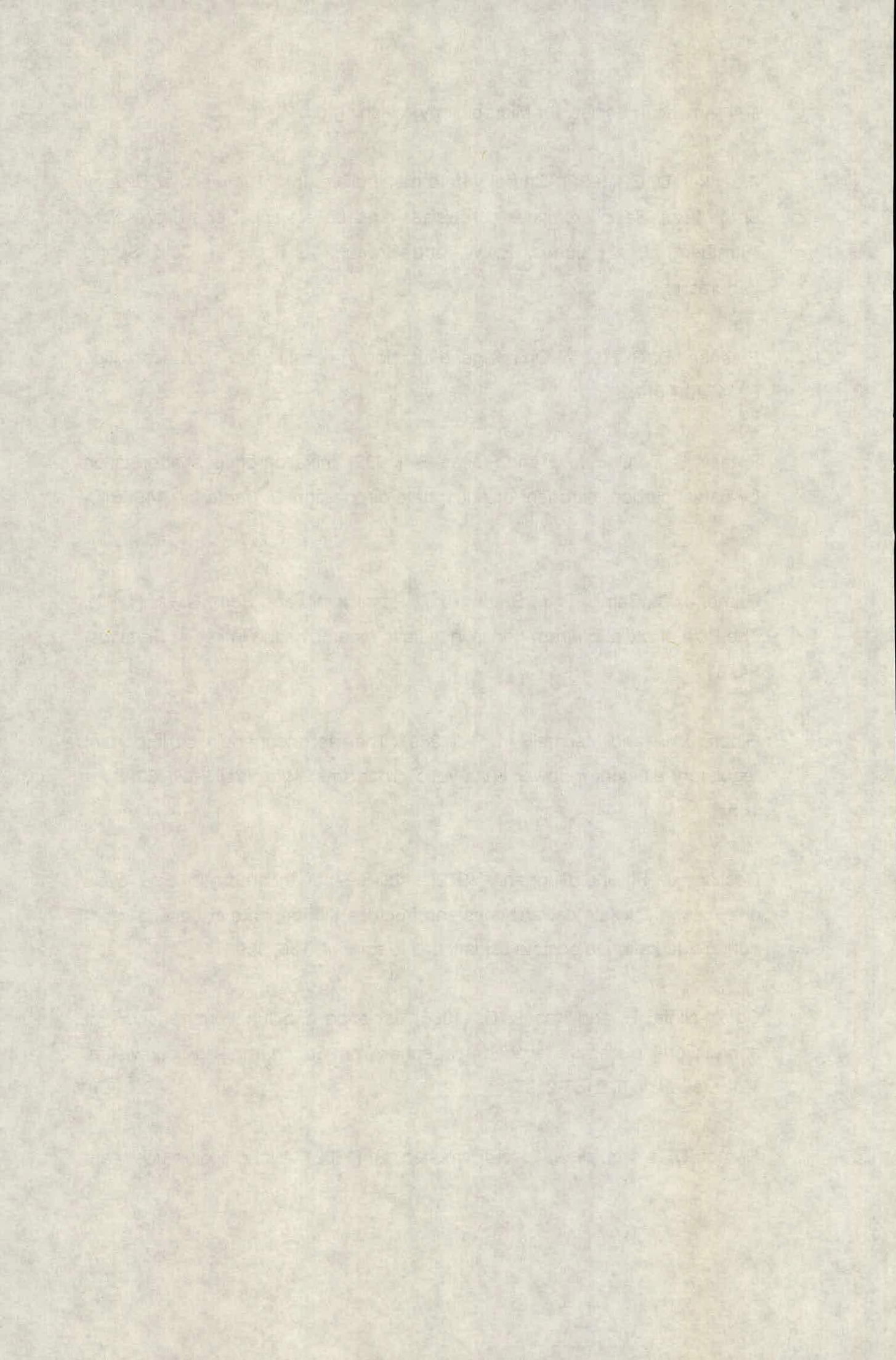
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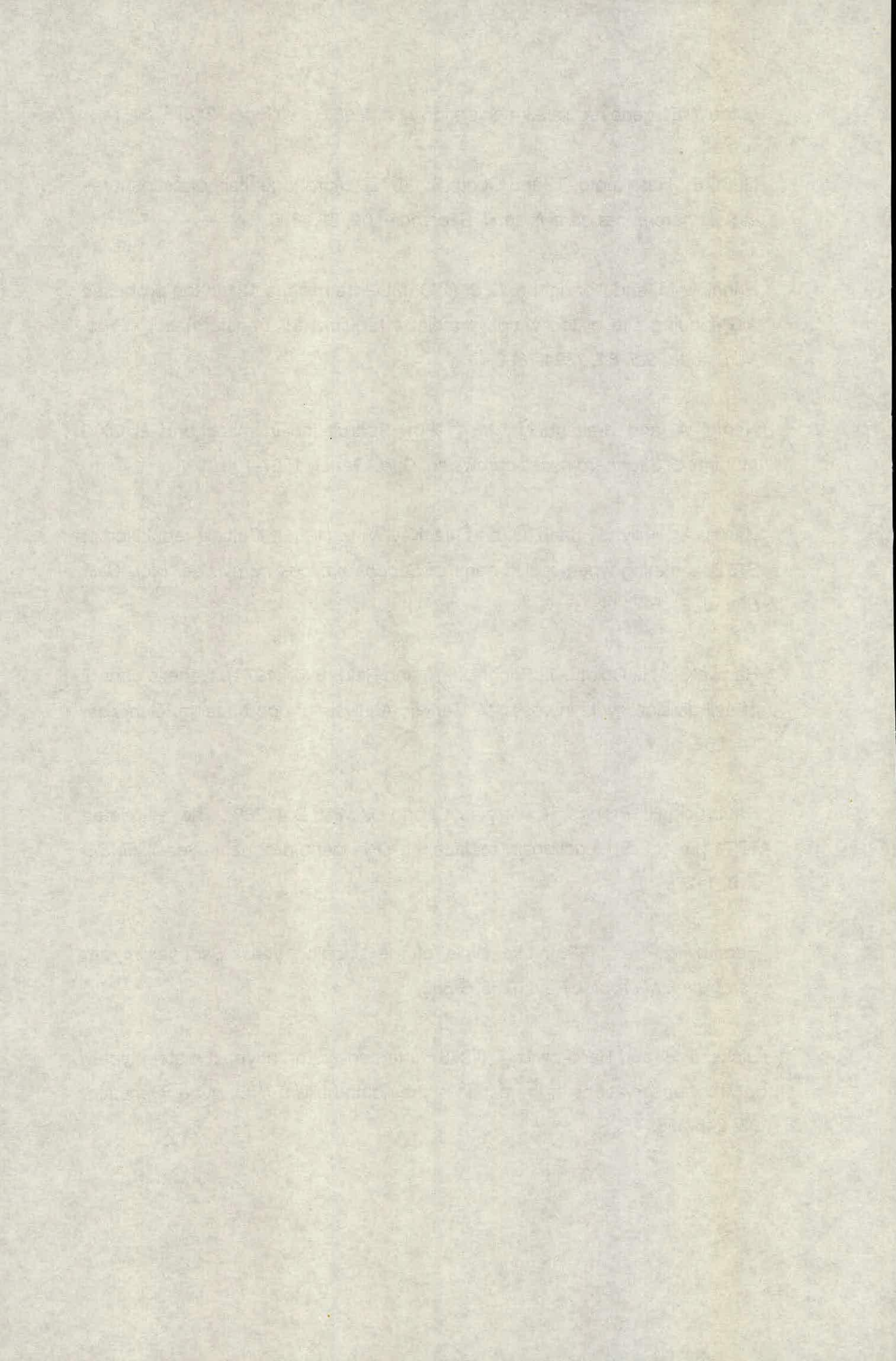
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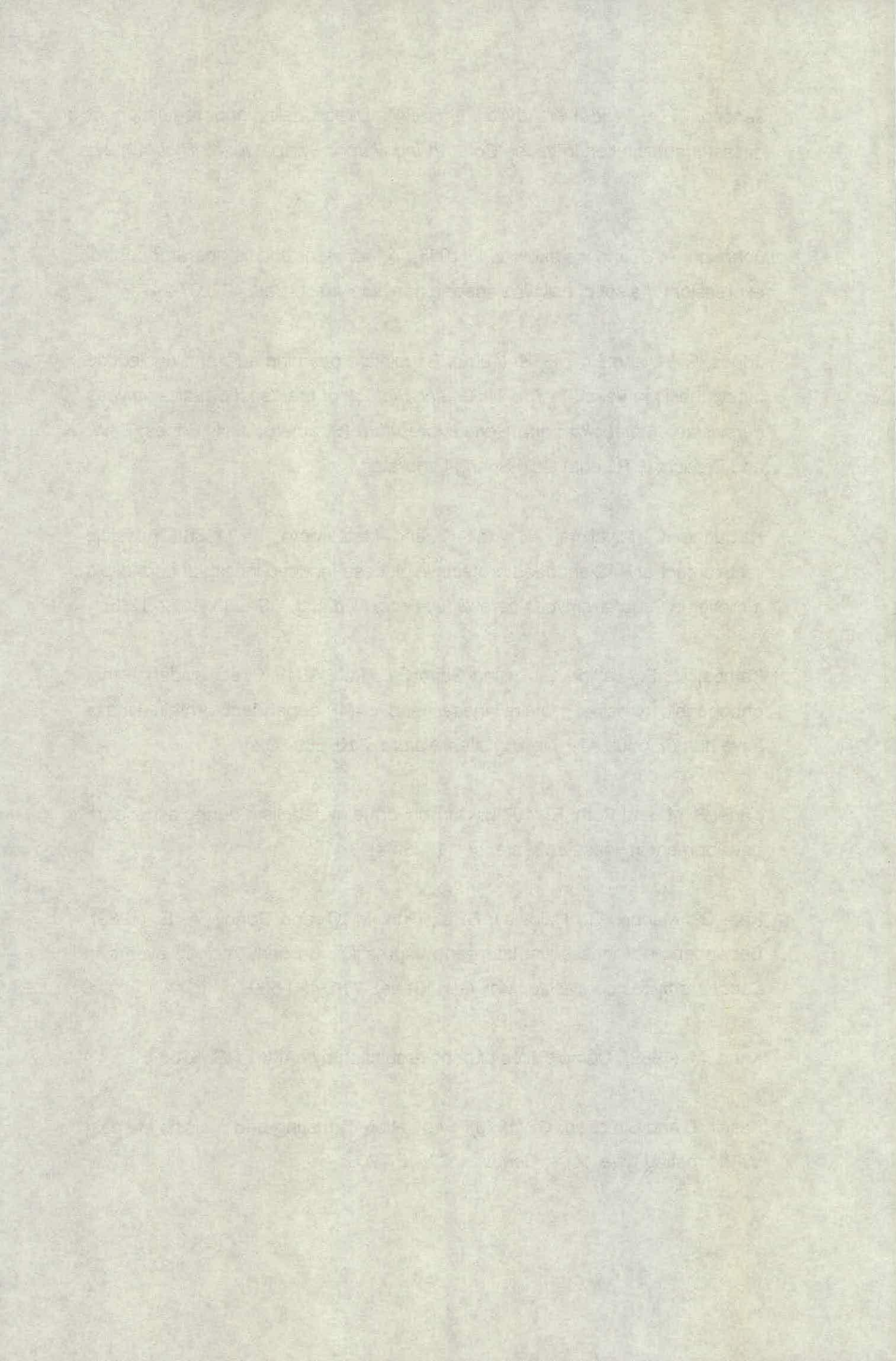
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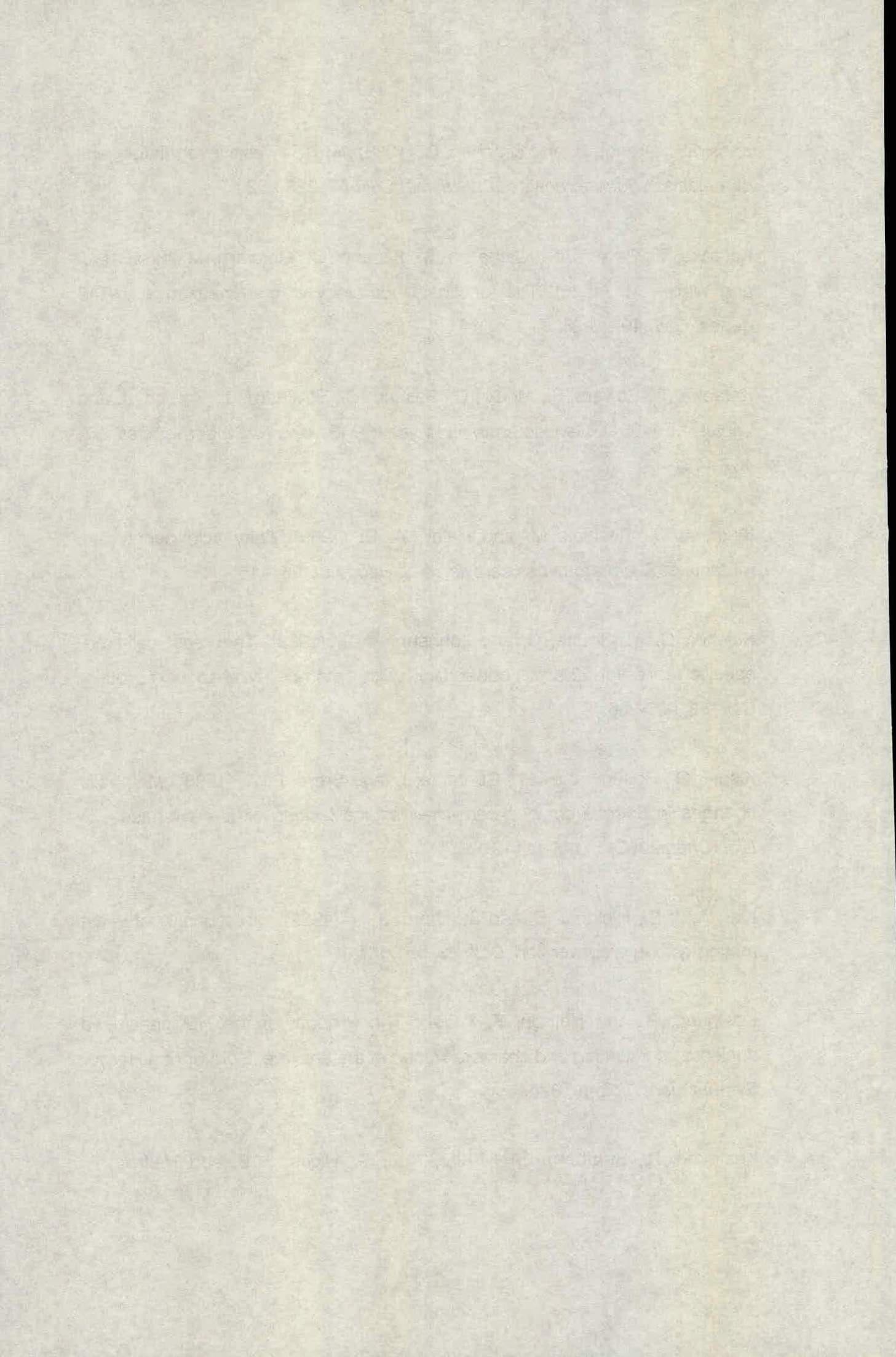
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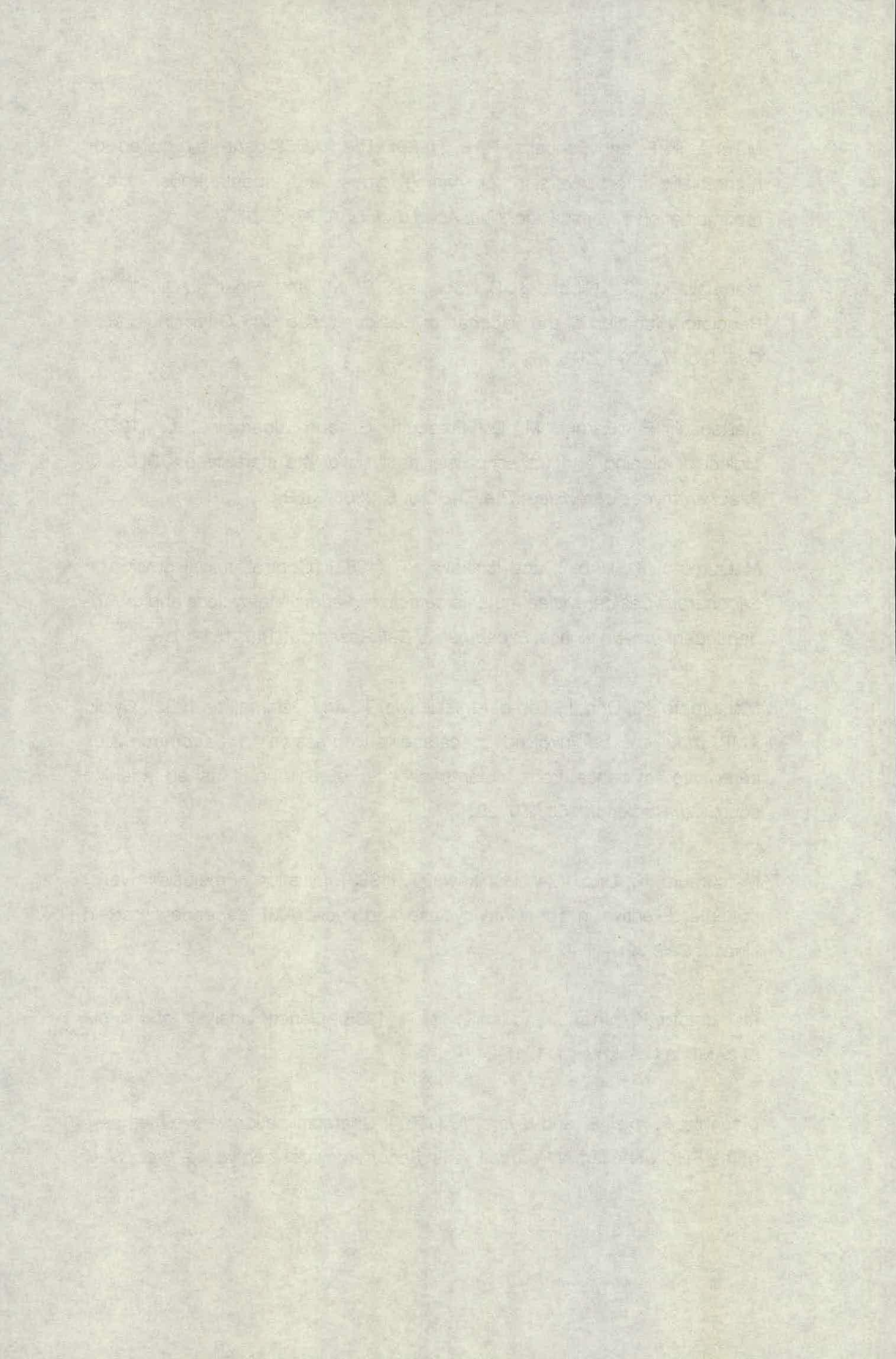
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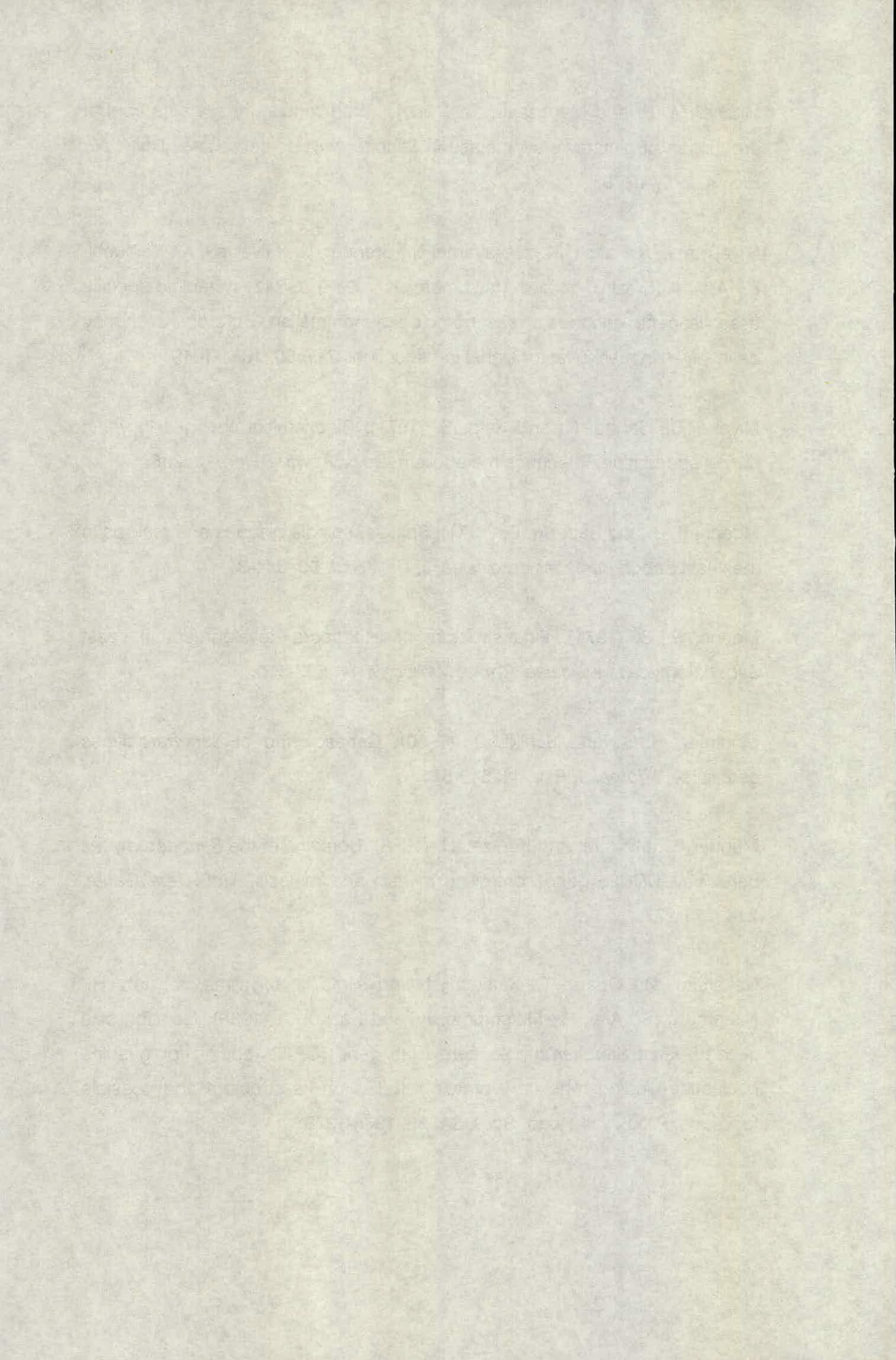
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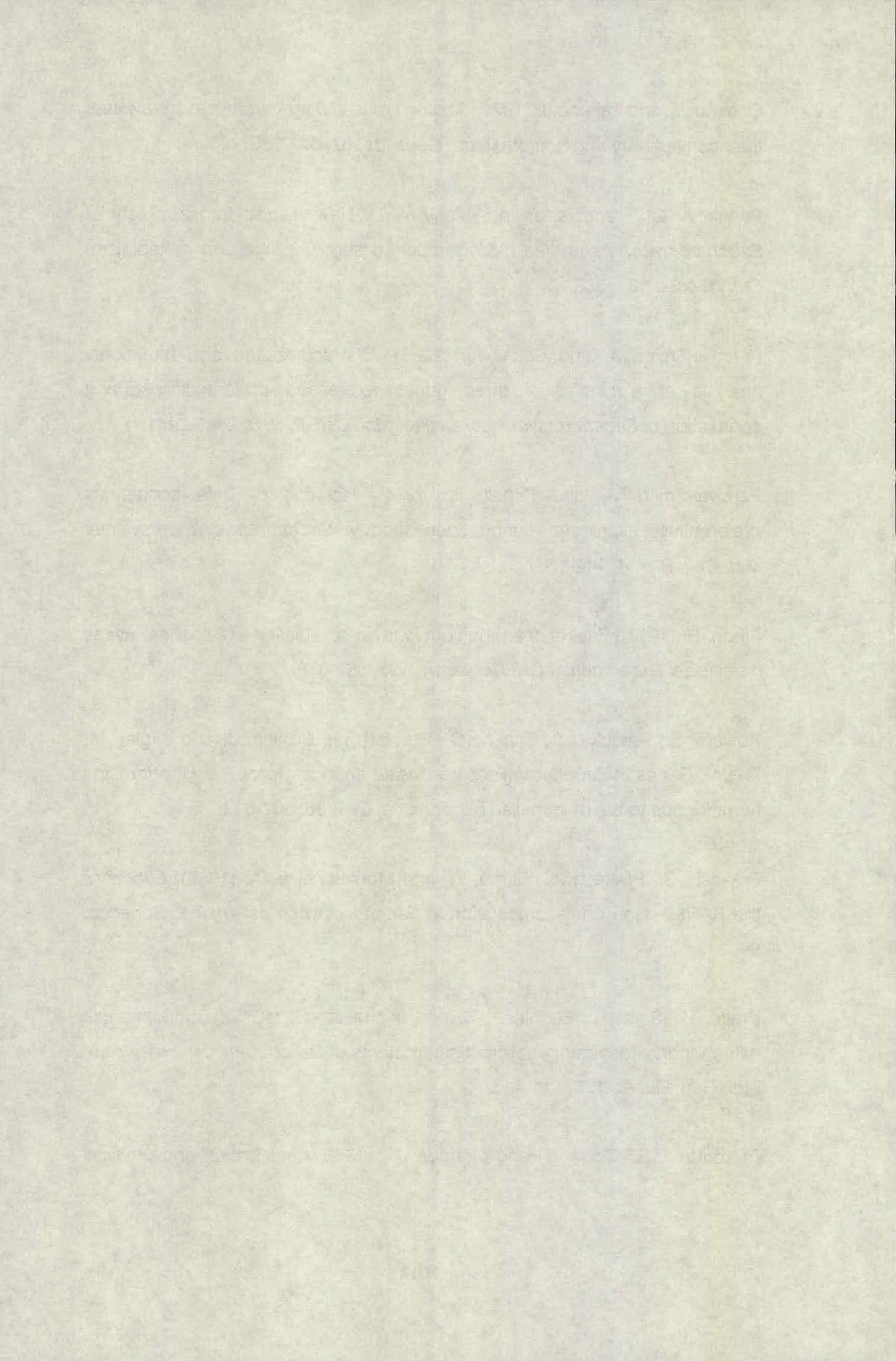
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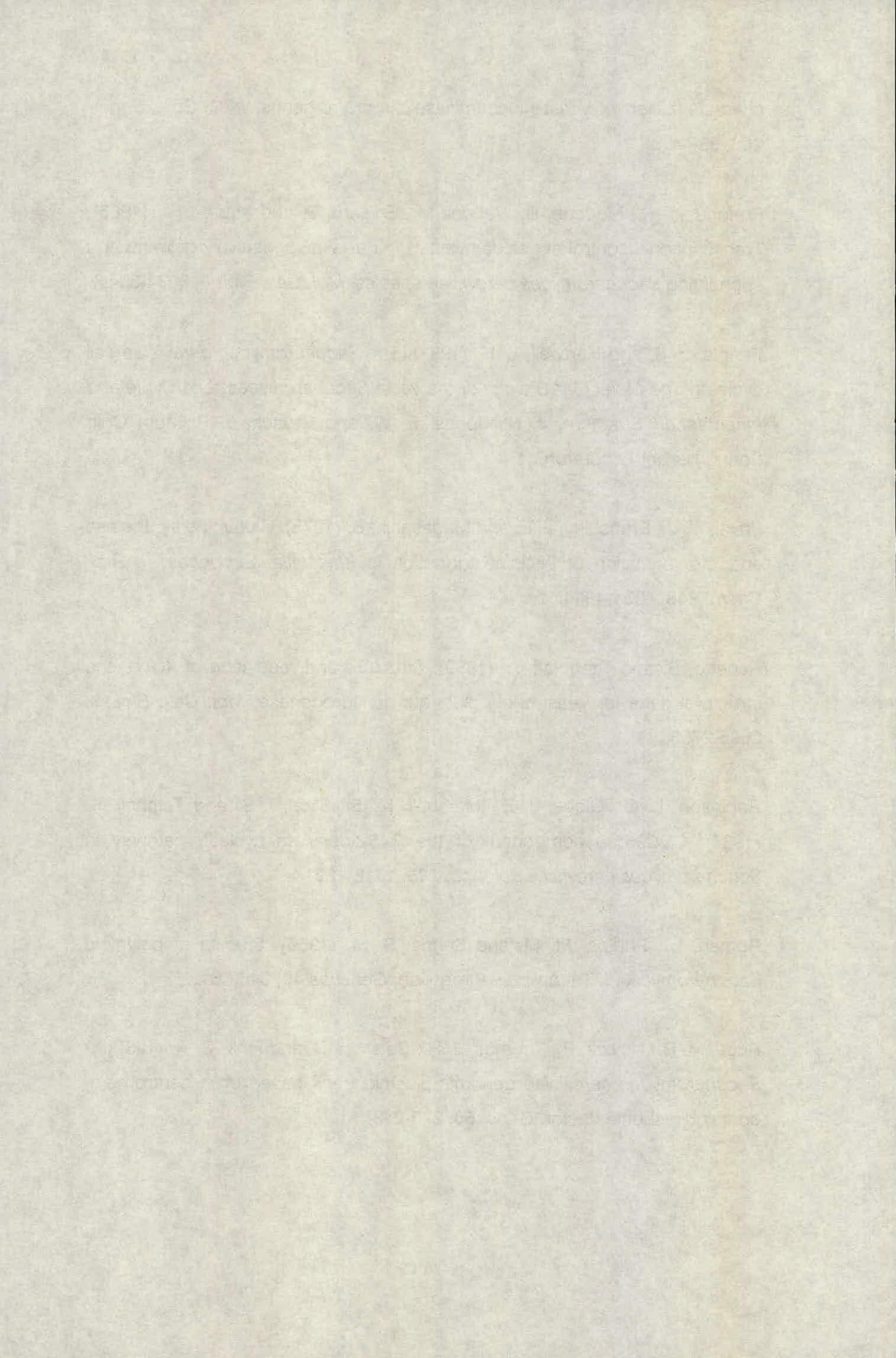
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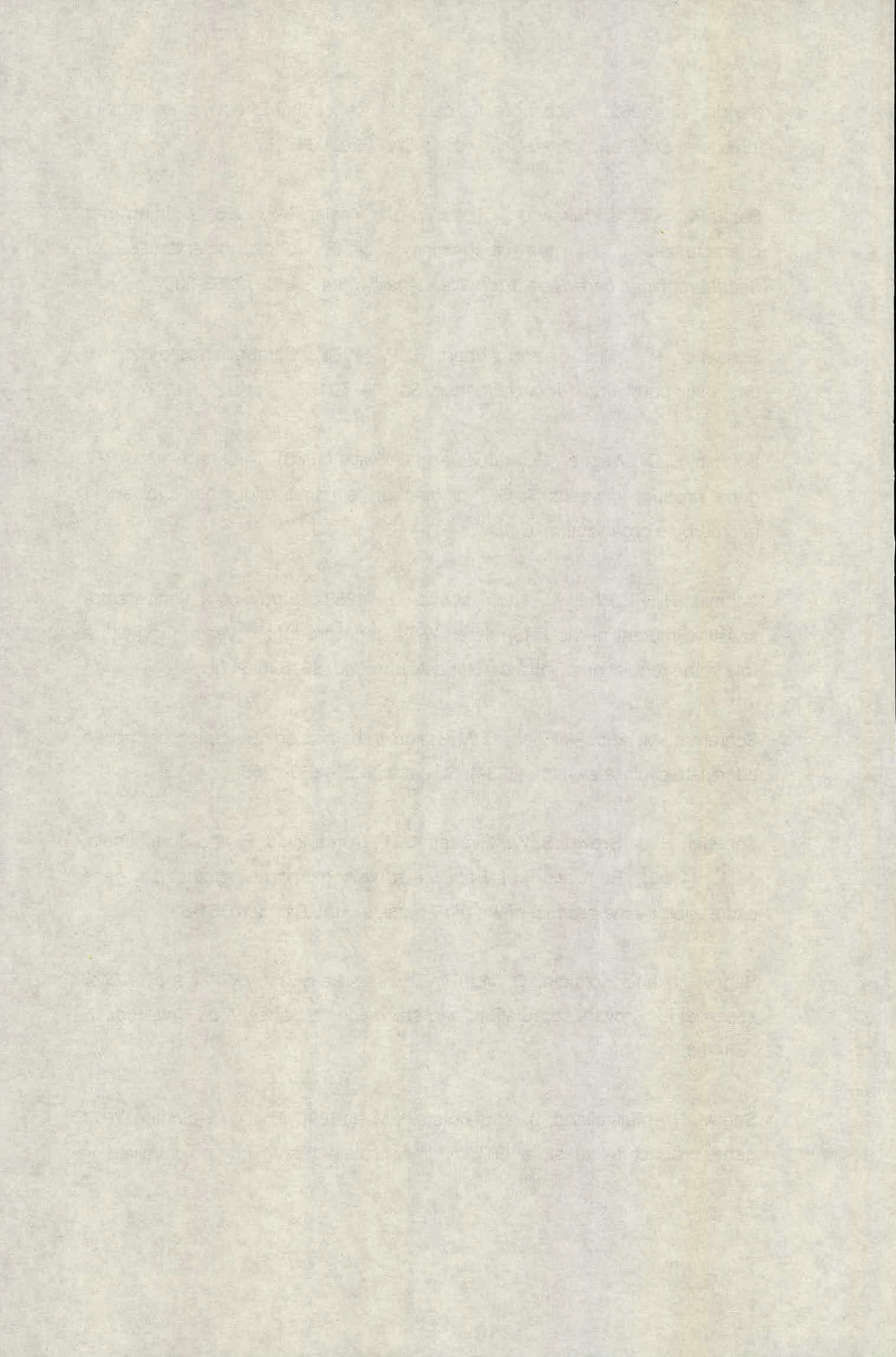
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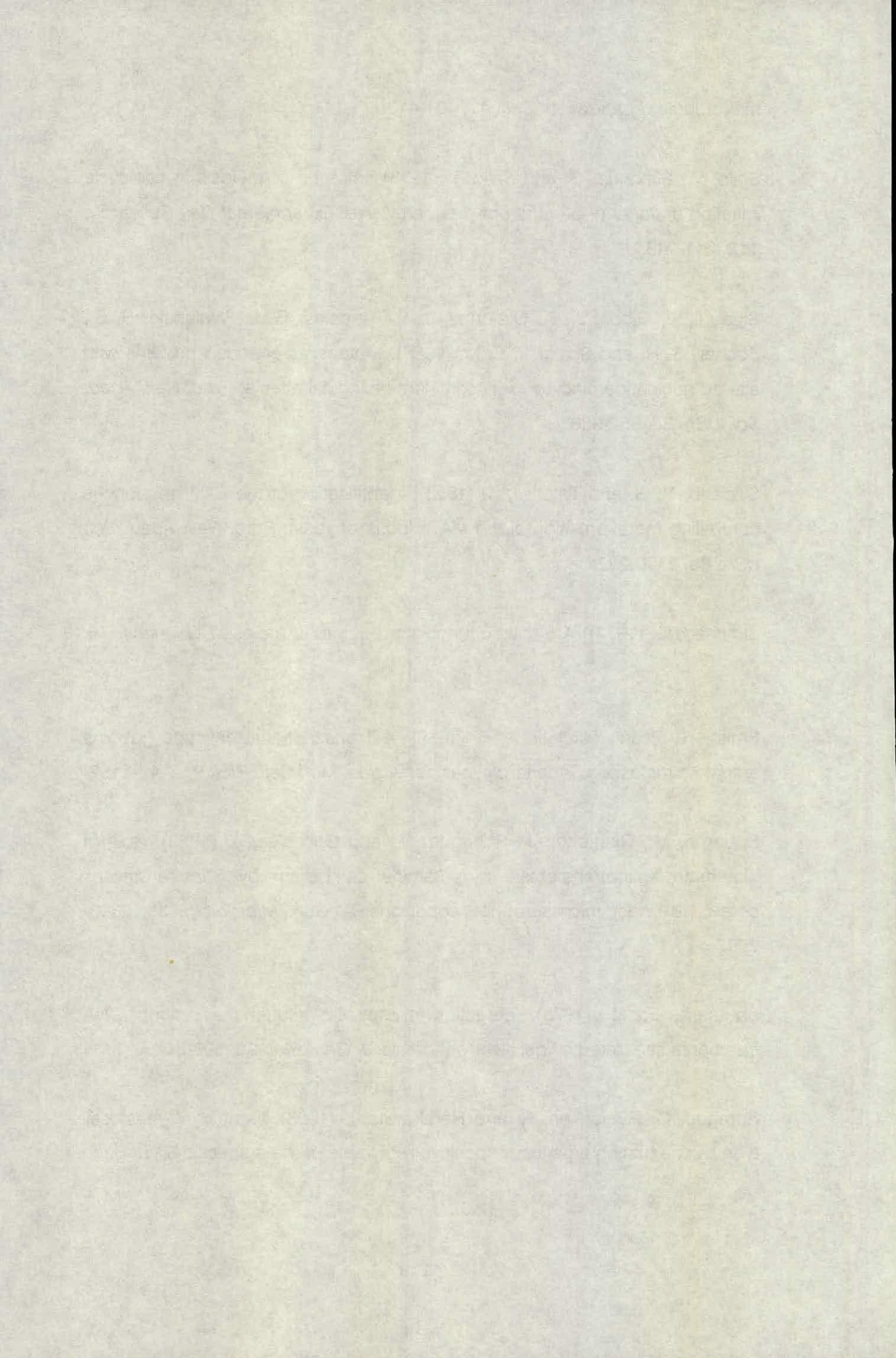
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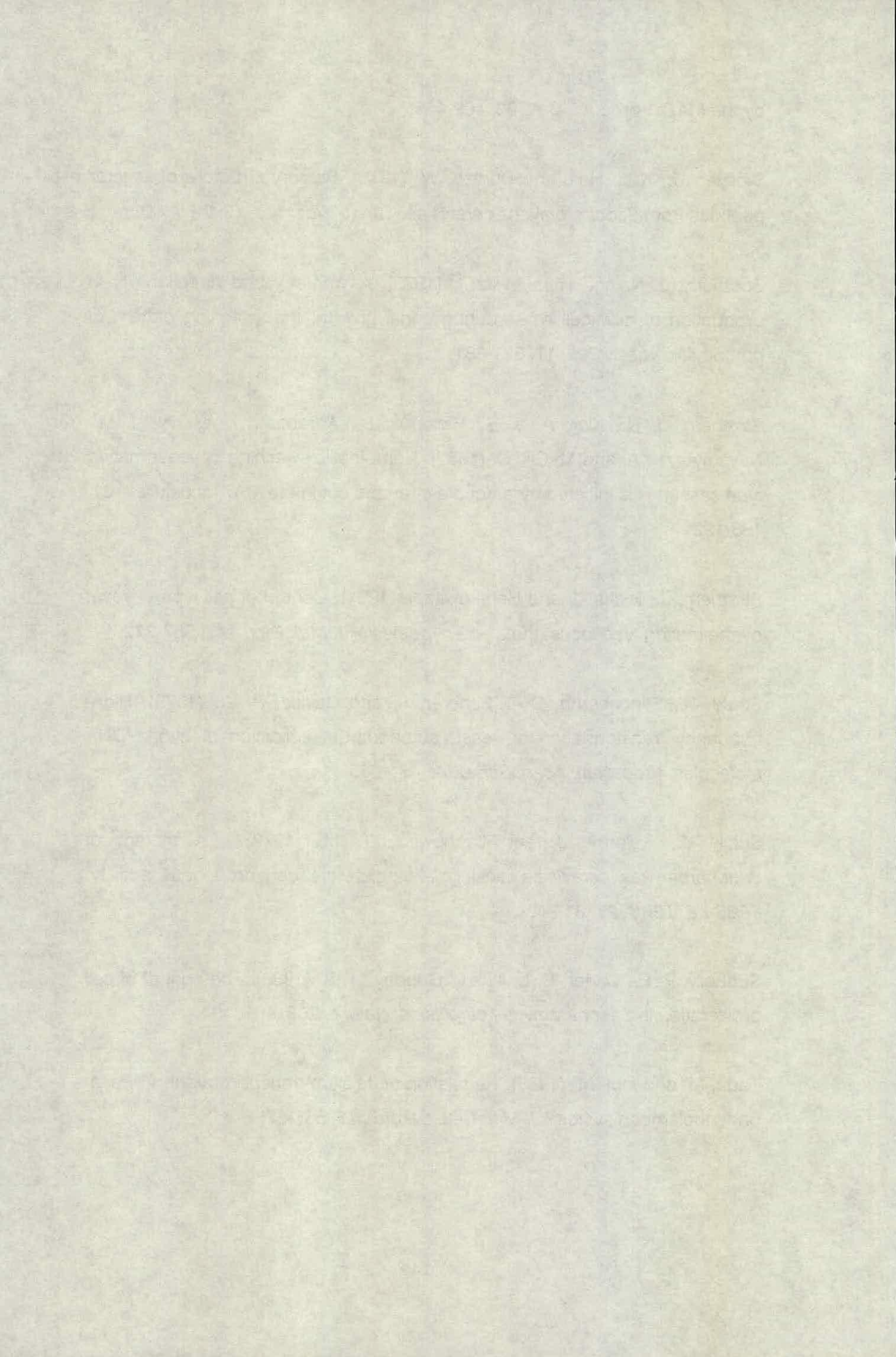
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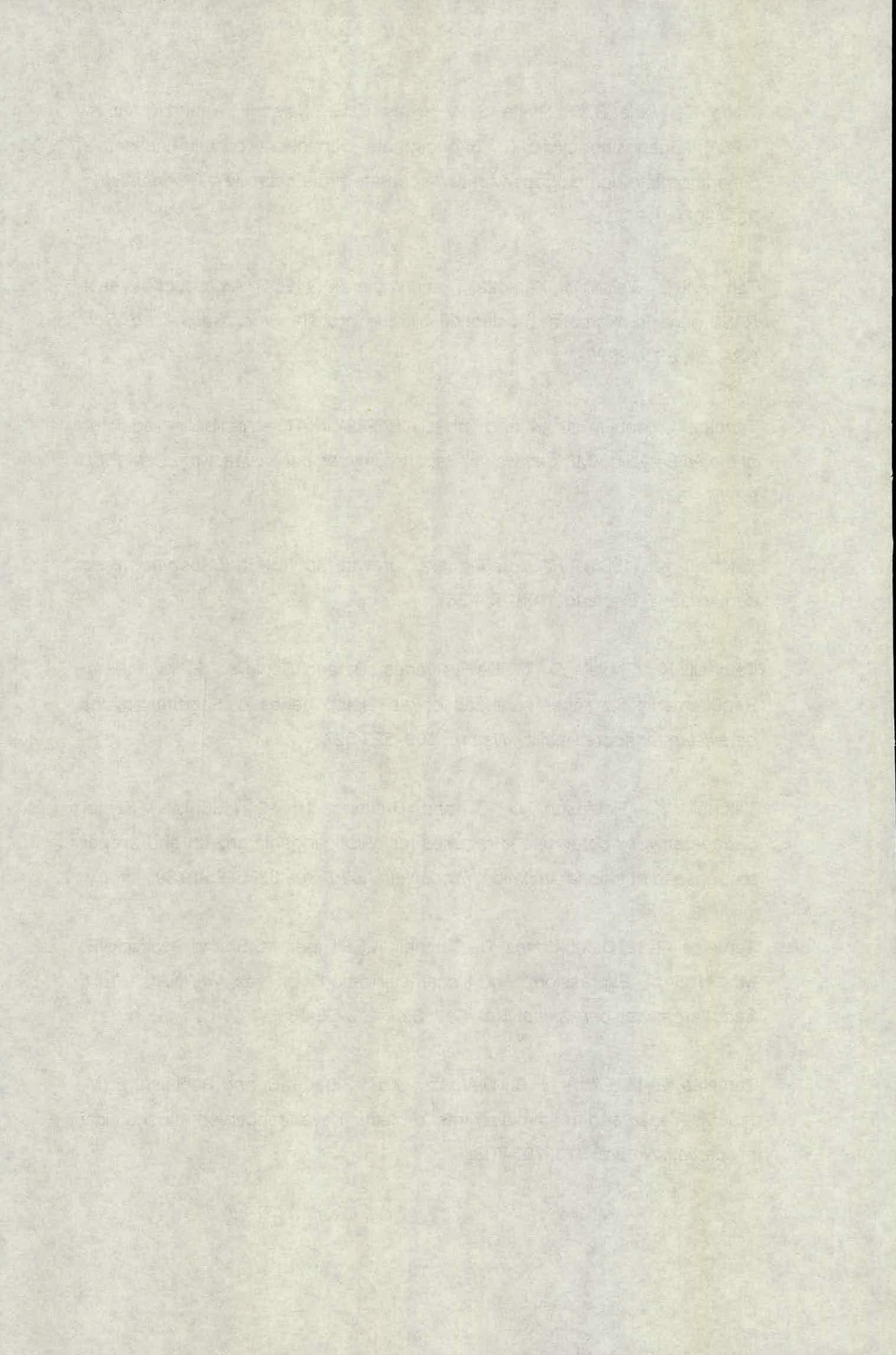
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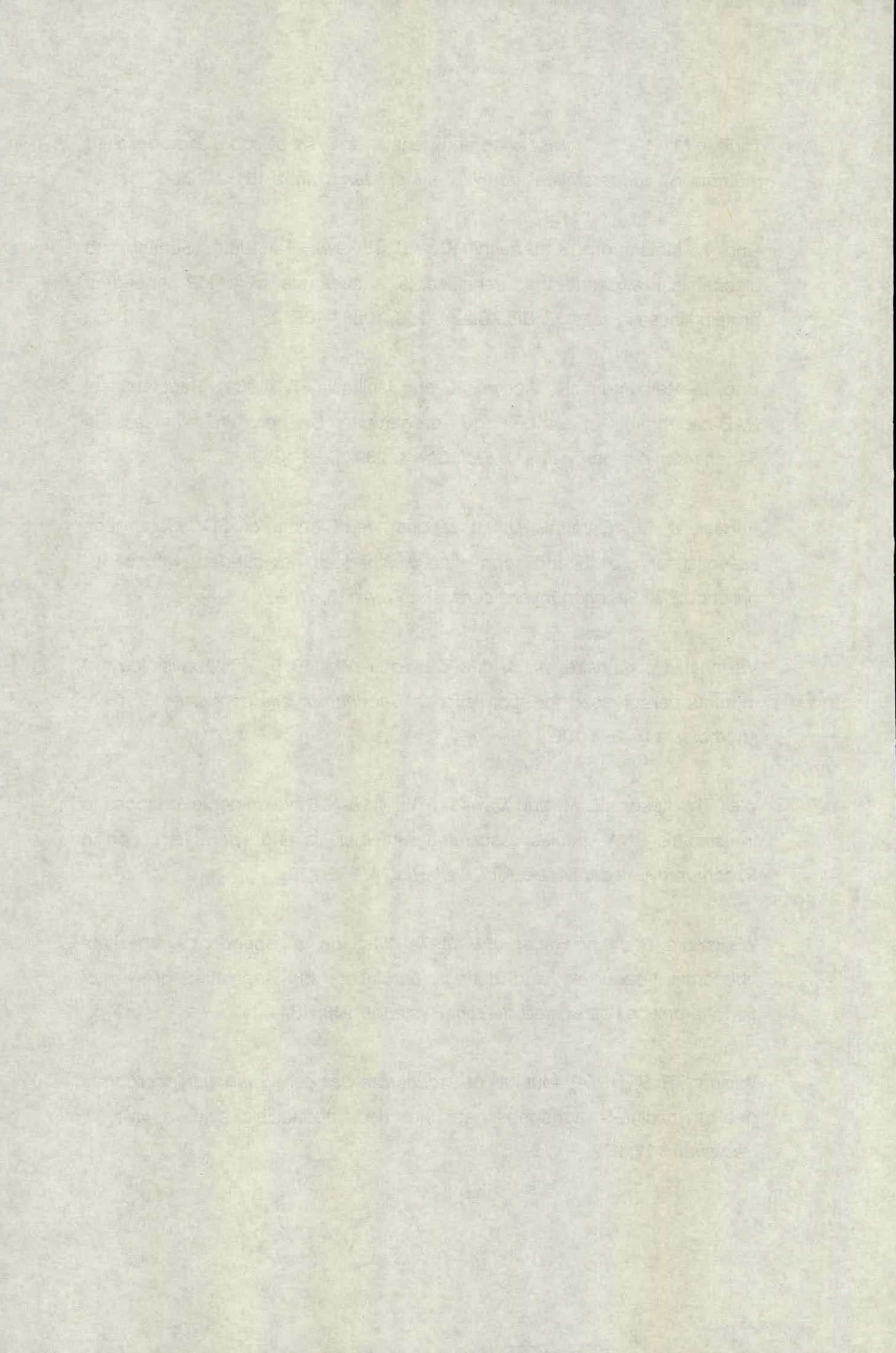
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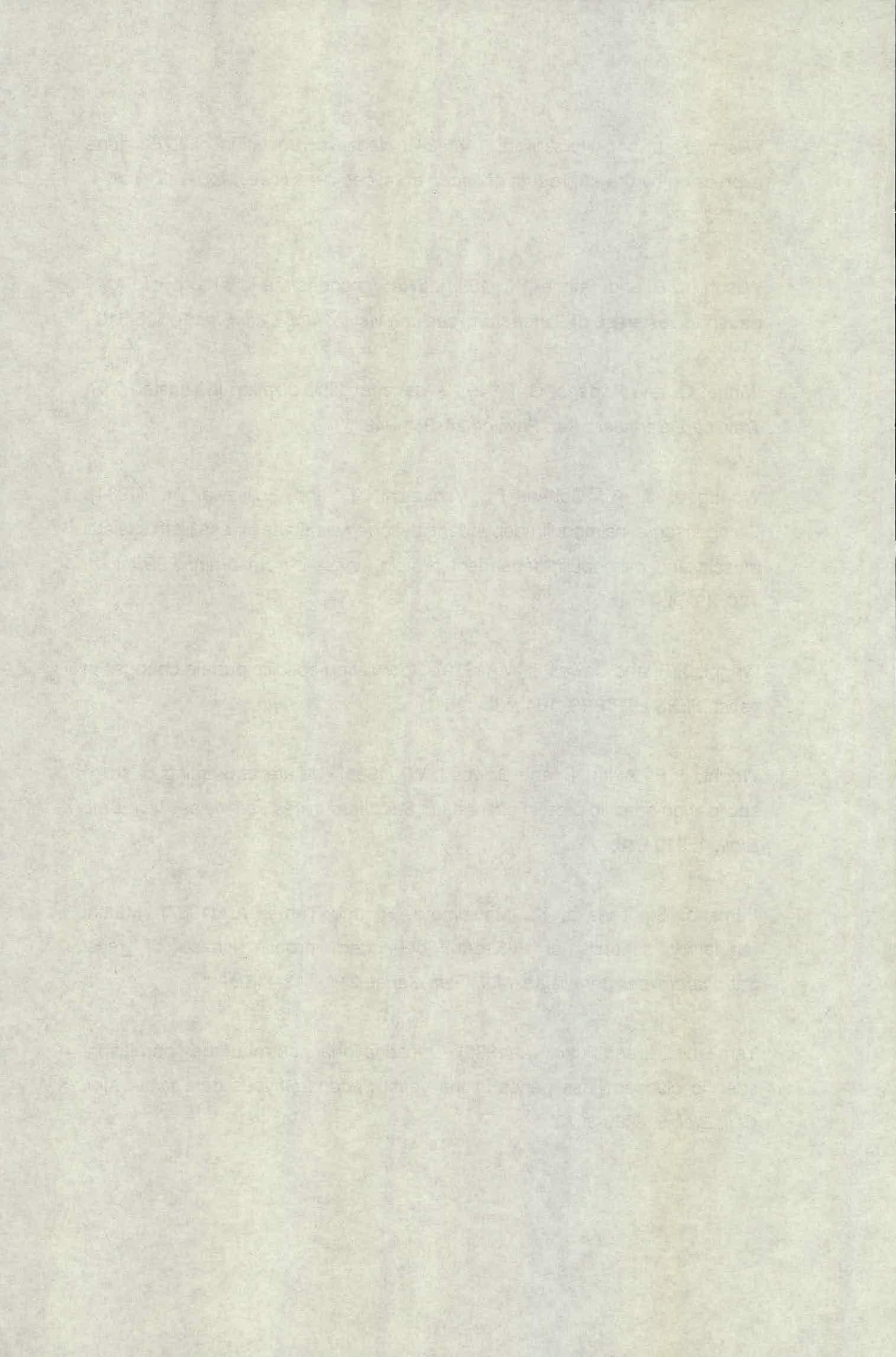
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